

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
14 June 2001 (14.06.2001)

PCT

(10) International Publication Number
WO 01/42284 A2

(51) International Patent Classification⁷: C07K 14/00 (81) Designated States (national): AE, AG, AI, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(21) International Application Number: PCT/GB00/04693 (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(22) International Filing Date: 7 December 2000 (07.12.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data: 9928950.6 7 December 1999 (07.12.1999) GB

(71) Applicant (for all designated States except US): METRIS THERAPEUTICS LIMITED [GB/GB]; 515 Eskdale Road, Winnersh, Wokingham, Berkshire RG41 5TU (GB).

(72) Inventor; and

(75) Inventor/Applicant (for US only): PAPPA, Helen [GB/GB]; Metris Therapeutics Limited, 515 Eskdale Road, Winnersh, Wokingham, Berkshire RG41 5TU (GB).

(74) Agents: MERCER, Christopher, Paul et al.; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).



WO 01/42284 A2

(54) Title: BINDING PROTEIN

FLT 316 GPSPKSVNTSVHIY 330DKAFITVKHRKQQVLE-TVAGKRSYRLSMKVKAFFSPEVVWLKDGLPATEKSARYLTR
KDR 312 GLMTKKNSTFVRVH 326EKFVAFGSGMESLVEATV-GER-VRIPAKYLGYPPPEIKWYKNGIP-LESN-HTIKV
FLK 314 GRMIKRNRTFVRVH 328TKPFIAGSGMKSILVEATV-GSQ-VRIPVKYLSPAPDIKWMYRNNGRP-IESNYTMV-V
FLT4 315 GIQRFRSTEVIVH 329ENPFISVEWLGPILEATA-GDELVLPVKAAYPPPFPQWYKDG-----KALSGRHS

FLT GYSLIIKDTBEDAGNYTILL--SI--KQSNVFKNLATLIVNVKPQIYEKAVSSFPD 440 PALYPLG447
KDR GHLVLTMEVSERDTGNYTILTNPISKEKQSHVV----SLVYVPPQIGEKSLSLISPVD 433 SYQY--G438
FLK GDELTIMEVTERDAGNYTILTNPISMEKQSHMV----SLVNVPPQIGEKALISPMD 435 SYQY--G440
FLT4 PHALVLKEVTEASTGTYTLALWNSAAGLR RNISLELVNVPPQIHEKEASSPS- 433 IYSR---437

Underlined:

Construct 0

(57) Abstract: The invention relates to novel compounds that act to prevent dimerisation of vascular endothelial growth factor (VEGF) receptors. The novel compounds may comprise the amino acid sequence of the fourth Ig-like domain of a VEGF receptor, or a variant that retains the ability to bind to a VEGF receptor. These compounds are useful in the inhibition of the biological activity of VEGF receptors and may thus be used to treat diseases in which VEGF plays a role.

BINDING PROTEIN

The present invention relates to novel proteins that act to prevent dimerisation of vascular endothelial growth factor (VEGF) receptors. These proteins are useful in the inhibition of the biological activity of VEGF receptors and may thus be used to treat diseases in which

5 VEGF plays a role.

VEGF is a potent stimulator of angiogenesis and plays an important role in the mammalian body in the development of the vascular system. It has been implicated in various human diseases such as inflammation, psoriasis, rheumatoid arthritis, hemangiomas, diabetic retinopathy, angiofibromas, macular degeneration, endometriosis, retinal 10 neovascularisation and cancer. The molecule has been implicated particularly in solid tumours, whose growth can be prevented by the inhibition of VEGF action (Kim *et al.*, (1993) *Nature* 362: 841-844)

VEGF plays a role in endometriosis (McLaren *et al.*, (1996) *Human Reproduction* 11, No.1, 220-223; McLaren *et al.*, (1996) *J. Clin. Invest.* 98 No.2, 482-489), the name given 15 to the disease that results from the presence of endometrium outside the uterine cavity. This disease affects women during their childbearing years with deleterious social, sexual and reproductive consequences. Endometriosis has been proposed as one of the most commonly-encountered diseases of gynaecology, with the incidence of endometriosis in the general population being estimated to be around 5%, although it is thought that at least 20 25% of women in their thirties and forties may be suffering from this disease.

The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and PLGF as well as their spliced variants. The biological activity of the VEGF family is mediated *in vivo* by three receptor tyrosine kinases that are primarily expressed in endothelial cells: kinase insert domain-containing receptor (KDR/FLK-1); the FMS-like 25 tyrosine kinase receptor-1 (FLT-1); and the FMS-like tyrosine kinase receptor-4 (FLT-4) which exhibit high binding affinity for the VEGF family members (de Vries *et al.*, (1992) *Science* 255: 989-991; Terman *et al.*, (1992) *Biochem. Biophys. Res. Commun.* 187: 1579-1586; Davis-Smyth *et al.*, (1996) *EMBO Journal* 15: 4919-4927). Additionally, the VEGF-A 165 isoform binds to neuropilin-1 (Soker S *et al.* (1998) *Cell* 92(6): 735-745) and 30 neuropilin-2 (Neufeld G *et al.* (1999) *FASEB J.*, 13(1): 9-22).

All of FLT-1, KDR/FLK-1 and FLT-4 are membrane-spanning receptors with an extracellular ligand-binding region containing seven immunoglobulin-like domains, a transmembrane domain and an intracellular tyrosine kinase domain. The transmembrane domain serves to anchor the receptor in the membrane of the cells in which it is expressed.

5 The biological activities of the FLT-1 and KDR receptors have been shown to differ, implying that these proteins have different functions *in vivo* (Roeckl *et al.*, (1998) Experimental Cell Research 241: 161-170; Shalaby *et al.*, (1995) Nature 376: 62-66; Fong G *et al.*, (1995) Nature 376: 66-70). FLT-4 is not a receptor for VEGF-A but rather binds to VEGF-C and VEGF-related protein (VRP). Like VEGF, VEGF-C and VRP can induce 10 mitogenesis in vascular endothelial cells, but at 100-fold less potency (Lee J *et al.*, (1996) Proc. Natl. Acad. Sci. USA 93: 1988-1992).

Various groups have investigated the structure of VEGF receptors in relation to the biological functions of these molecules. Keyt *et al.*, (1996) (J. Biol. Chem. 271 (10) 5638-5646) mapped the residues important for VEGF binding on both KDR and FLT-1 and 15 suggested that VEGF displays different receptor binding sites for KDR and FLT-1.

Shinkai *et al.*, (1998) (Journal of Biological Chemistry 273 (47): 31283-31288) mapped various sites of the extracellular domain of the KDR receptor thought to be involved in ligand association and disassociation and concluded that the third Ig-like domain is critical for ligand binding with the second and fourth domains playing a role in ligand association. 20 The fifth and sixth domains are required for retention of the ligand when bound to the receptor molecule, while the first Ig-like domain was proposed to regulate ligand binding.

Davis-Smyth *et al.*, (1996) (EMBO Journal 15 (18): 4919-4927) reported that the second Ig-like domain of the FLT-1 receptor contains critical determinants of ligand binding. Furthermore, when the FLT-4 domain 2 was exchanged for that of FLT-1, FLT-4 became 25 non-responsive to its natural ligand VEGF-C, suggesting that domain 2 is also critical for binding in the FLT-4 receptor. These findings suggested that determinants for binding and ligand specificity within the second Ig-like domain is a common feature of subclass III receptor tyrosine kinases with seven Ig-like domains. Later studies proved that domains 2 and 3 are necessary for ligand binding with wild type affinity (Barleon *et al.*, (1997) J. Biol. Chem. 272 (16): 10382-10388; Davis-Smyth *et al.*, (1998) 273 (6): 3216-3222; Wiesmann *et al.*, (1997), Cell 91: 695-704). The ligand-FLT-1 domain 2 interactions were 30

determined in detail by the determination of the high resolution structure of FLT-1 domain 2 with VEGF (Wiesmann *et al.*, 1997, *Cell* 91; 695-704).

In an independent study, Barleon *et al.* (1997) (*J. Biol. Chem.* 272 (16); 10382-10388) mapped the sites for ligand binding and receptor dimerisation in the extracellular domain 5 of the FLT-1 receptor and confirmed that the first three Ig-like loops are involved in high affinity binding of VEGF. Dimerisation of the extracellular domains of FLT-1 receptor was only detected in the constructs that contain the fourth Ig-like loop.

Kendall & Thomas, 1993 (P.N.A.S. USA 90; 10705-10709) reported the cloning of a soluble truncated form of FLT-1 from a human vascular endothelial cell library. This 10 molecule was found to comprise the six N terminal immunoglobulin-like extracellular ligand-binding domains but to lack the transmembrane-spanning region and intracellular tyrosine kinase domains. Binding affinity for VEGF-A was retained, prompting these workers to speculate that this soluble receptor might act as an efficient specific antagonist of VEGF *in vivo*.

15 Clark *et al.*, (1998) (*Biol. Reprod.* 59: 1540-1548) reported the occurrence of soluble FLT-1 (sFLT-1) in serum from pregnant women, which was not present in serum from men and from non-pregnant women. These workers thus speculated the *in vivo* production of the FLT-1 receptor might constitute a mechanism for naturally-regulating VEGF-induced angiogenesis. No naturally-occurring secreted form of KDR has been reported to date, 20 however, sFLT-1 has been shown to form ligand-induced heterodimeric complexes with full length KDR (Kendall *et al.*, (1996), *Biochem. Biophys. Res. Commun.* 226 (2): 324-328). To the best of the Applicant's, there is no naturally-occurring secreted form of FLT-4.

Certain approaches have been suggested that attempt to treat VEGF mediated disease by 25 supplying VEGF antagonists such as neutralising antibodies, VEGF receptor molecules, and portions of such receptor molecules (see, for example co-pending patent application PCT/GB95/01213, Metris Therapeutics; PCT/US97/17044, Merck & Co., Inc.; PCT/US97/07694, Genentech, Inc.; PCT/US92/09218, Genentech, Inc.; PCT/US94/01957, Merck & Co., Inc.). However, all of the approaches embodied in these patent applications 30 rely on reducing the effective concentration of VEGF molecules, and none of the suggested approaches have yet provided agents that are effective against all types of VEGF-mediated disease.

There thus remains a great need for novel compounds that are effective to disrupt VEGF function *in vivo*.

Summary of the Invention

According to a first aspect of the invention there is provided a protein consisting of the 5 amino acid sequence of the fourth Ig-like domain of a VEGF receptor, a variant of said protein that retains the ability to bind to a VEGF receptor or a functional equivalent of said fourth Ig-like domain.

All VEGF receptors form homodimers. The VEGF molecule itself acts as a dimer, and the binding of one monomer component to a receptor molecule induces the dimerisation of the 10 VEGF receptor molecule through the interaction of the second monomer component with a second VEGF receptor in the cell membrane (Fuh *et al.*, 1998, *J. Biol. Chem.* 273, No.18, 11197-11204). The dimerisation of the VEGF receptor molecule induces the activation of the intracellular kinase domain of the receptor, thus initiating the signal transduction cascade that is effective to translate the ligand-receptor binding event into the activation of 15 the appropriate secondary messenger system in the cell.

The present invention provides molecules that when bound to the fourth Ig-like domain of a full length VEGF receptor, prevent its dimerisation. Since VEGF-dependent activation of the intracellular signalling domain of the full-length receptor occurs only when the VEGF receptor is in its dimeric state, blocking the dimerisation event severs the link between 20 ligand binding and activation of the secondary messenger system. Accordingly, the biological action of VEGF may be specifically blocked.

The invention has a number of advantages over systems that have been previously described. Most of these systems involve mechanisms that are designed to remove the effective amount of VEGF from circulation, either systemically, or in specific areas of the 25 body. Such techniques are far from ideal for a number of reasons, the most obvious being that VEGF is a molecule with a wide range of biological functions in the body. Lowering the effective levels of this molecule, either by preventing its expression, or by interfering with it directly through specific binding events, acts to abolish VEGF function altogether, so leading to unwanted side-effects. Studies that have used small molecule inhibitors to 30 target VEGF-Receptor tyrosine kinases may inhibit other kinases and so cause unwanted side-effects.

One advantage of using the molecules of the present invention in therapy is that VEGF itself is left unaffected, so a free population of the VEGF molecule remains to perform its natural biological functions. By targeting the receptor molecules themselves, VEGF levels remain unchanged, meaning that the normal VEGF-mediated processes are allowed to 5 continue unaltered by the therapy process. Furthermore, the molecules of the present invention may be designed to target only a subset of VEGF receptor types, so leaving non-targeted receptors unaffected, meaning that this method of therapy is unlikely to cause undesirable side-effects.

By the term the "fourth Ig-like domain" is meant the immunoglobulin-like domain of the 10 VEGF receptor that is considered by the inventors to be necessary for the dimerisation function of the receptor molecule. This domain is defined as being the fourth Ig-like domain as counted from the NH₂ terminus of the receptor molecule. It is not at present clear which precise residues participate in the dimerisation event. However, the molecules of the invention should retain sufficient residues from the fourth Ig-like domain to bind to 15 the corresponding domain of a VEGF receptor with high enough affinity to compete effectively for binding with wild type full length VEGF receptor molecules.

At present, there are three VEGF receptors known. However, the present invention is likely to be equally applicable to other VEGF receptors that are discovered in the future. Preferably, the proteins of the present invention are derived from the receptors FLT-1, 20 FLK/KDR and FLT-4. The VEGF receptors to which the proteins of the invention bind are preferably mammalian, most preferably human VEGF receptors.

In FLT-1, the boundaries of the fourth Ig-like domain are considered to be within amino acid residues 316 or 317 and 447 inclusive, wherein the numbering system starts at the first methionine residue in Figure 1. However, shorter protein molecules may be used, provided 25 that the molecules include at least amino acid residues 344-406 of the FLT-1 sequence. Preferably, those proteins of the invention that are derived from FLT-1 consist of at least residues 338-440, more preferably 330-440 of the full length FLT-1 sequence. Examples of particularly preferred constructs include those that consist of residues 330-440, 330-429 or 338-429 of the full length FLT-1 sequence.

30 The analogous residues in the FLK receptor are shown in Figure 5. The boundaries of the fourth Ig-like domain of this receptor is considered to be at residues 314 and 440 of the full length FLK sequence given in Figure 2. At the very least, this domain should include

residues 342-404 of the FLK sequence. Preferably, those proteins of the invention that are derived from FLK consist of at residues 336-439, 335-435, 335-424, more preferably residues 328-424 or 328-435 of the full length FLK sequence.

The analogous residues in the KDR receptor are also shown in Figure 5. The boundaries of 5 the fourth Ig-like domain of this receptor are considered to be at residues 312-438 of the full length sequence given in Figure 3. At the very least, this domain should include residues 340 to 402 of the KDR sequence. Preferably, those proteins of the invention that are derived from KDR consist of at residues 333-438, 333-433 or 333-422, more preferably residues 326-422 or 326-433 of the full length KDR sequence.

10 The analogous residues in the FLT-4 receptor are also shown in Figure 5. The boundaries of the fourth Ig-like domain of this receptor are considered to be residues 315 to 437 of the full length sequence given in Figure 4. At the very least, this domain should include residues 343-403 of the FLT-4 sequence. Preferably, those proteins of the invention that are derived from FLT-4 consist of residues 339-437, 339-423, more preferably, 329-423 or 15 329-437 of the full length FLT-4 sequence.

The boundaries of the fourth Ig-like domain were predicted, based on sequence alignments of FLT-1 and KDR with telokin, as well as secondary structure predictions assisted by the crystal structure of the telokin molecule (PDB file: 1TLK).

The maximum boundaries of the fourth Ig-like domain were determined to reside within a 20 few residues of the last conserved cysteine residue in the third Ig-like domain, and the first conserved cysteine residue of the fifth Ig-like domain (Cys311 and Cys454 respectively).

The minimum boundaries were defined after aligning FLT-1 to telokin and defining the minimum number of secondary structure elements that would be sufficient to support a stable protein fold.

25 Additionally, information of the exon-intron genomic organisation of FLT-1 was generated by the Applicant, constructed from sequencing data generated from the human genome sequencing effort. FLT-1 resides within chromosome 13; the genomic organisation of this gene was deciphered using recently deposited sequencing data of chromosome 13 clones.

The identification of the appropriate boundaries of other VEGF receptors to which the 30 teaching of the present invention may be applied will be clear to those of skill in the art. Details of the boundaries of other preferred constructs are shown in Table 1.

The proteins of the present invention may comprise an amino acid sequence that corresponds exactly to the wild type receptor protein sequence found in the fourth Ig-like domain of the VEGF receptor protein. The wild type amino acid sequences of the fourth Ig-like domains of the FLT-1, FLK/KDR and FLT-4 receptors are shown in Figure 5.

5 However, as the skilled reader will appreciate, the proteins of the invention may be derived from any mammalian VEGF receptor sequence. Human sequences are preferred.

As used herein, the term "wild type" means the amino acid sequence that is characteristic of most of the members of the particular species from which the receptor molecule is derived. Included within the term "wild type" are natural biological variants of the VEGF 10 receptor molecule sequences (for example, allelic variants or geographical variations within the species from which the wild type proteins are derived).

The proteins of the present invention may most suitably be derived from VEGF receptors in the FLT-1, KDR/FLK and FLT-4 receptor family. All of these molecules dimerise through the interaction of the respective fourth Ig-like domains of the molecule. Of 15 particular applicability to the present invention are recombinant proteins derived from the FLT-1 and KDR/FLK proteins. These molecules, and the effect of these molecules when aberrantly-regulated or when mutated, is thought to have a particularly important role in the pathology of diseases such as cancer and endometriosis.

Variants of wild type fourth Ig-like domain receptor sequences are also included in the 20 present invention. As the skilled man will appreciate, the term "variant" includes molecules that contain single or multiple amino-acid substitution(s), addition(s), insertion(s) and/or deletion(s) from the wild type protein sequence, provided that such variants maintain the ability to bind to the corresponding fourth Ig-like domain of target VEGF receptor and thus prevent their dimerisation. Variant molecules may also contain substitutions of chemically- 25 modified or synthetic amino acids that do not affect the function or activity of the protein in an adverse manner.

Suitable variants of the molecules of the invention will be those proteins that exhibit high affinity for the fourth Ig-like domain of a VEGF receptor. Preferably, this affinity is higher than that of the wild type sequence. Typically, the protein according to the present invention 30 binds to a VEGF receptor with a dissociation constant of 2 μ M or less, preferably, 0.2 μ M or less, more preferably 2nM or less, even more preferably, 20pm or less.

Another property that is desirable for a variant of the wild type sequence is the ability to bind to the fourth Ig-like domain of an intact VEGF receptor with a significantly higher affinity than the wild type protein displays for binding to the intact VEGF receptor. The dimerisation kinetics will lead towards the formation of heterodimers with target receptor with increasing

5 concentrations of the proteins of the invention. Such concentrations will vary for different cell types as they will depend on the number of target receptor molecules that are present on the cell surface. However, a large excess of protein should be able to mask all cell surface receptors, so conferring a therapeutic effect to the patients to whom preparations of these proteins will be administered.

10 The term "functional equivalent" is used herein to describe proteins that have an analogous function to the fourth Ig-like domain of a VEGF receptor and that bind specifically to this domain, thus preventing dimerisation of the receptor molecules and so inhibiting signal transduction effected by ligand binding to the VEGF receptor. This term therefore includes molecules that are structurally similar to the fourth Ig-like domains identified herein or that

15 contain a similar or identical tertiary structure. The analogous binding properties of functional equivalents should be reflected in their affinity for the fourth Ig-like domain of a VEGF receptor. Typically, functional equivalents should bind to a VEGF receptor with a dissociation constant of $2\mu\text{M}$ or less, preferably, $0.2\mu\text{M}$ or less, more preferably 2nM or less, even more preferably, 20pm or less. For these molecules, the thermodynamics of

20 binding should be sufficient that physiologically-attainable concentrations of molecule are effective to prevent VEGF receptor dimerisation.

The term "functional equivalent" therefore includes entities such as antibodies, (particularly antiidiotypic antibodies), oligopeptides, peptides, peptidomimetics, drug molecules such as small natural or synthetic organic molecules of up to 2000Da , preferably

25 800Da or less in size. Other examples of functional equivalent molecules will be clear to those of skill in the art.

Functionally-equivalent peptide, oligopeptide or polypeptide compounds according to the present invention may be generated by any suitable means, as will be apparent to those of skill in the art. In addition to the naturally-occurring amino acids, these molecules may, of

30 course, contain synthetic amino acids.

In the case of antiidiotypic antibodies, these may be obtained by immunisation of an appropriate host with a preparation of an antibody that recognises the dimerisation interface within the fourth Ig-like domain molecule.

In the case of peptides, combinatorial peptide libraries may be most suitable to isolate 5 peptide molecules that display the desired binding characteristics, through the use of selection regimes that select for molecules that bind to antibodies that are specific for the fourth Ig-like domain of a VEGF receptor.

One method of generation of peptide libraries utilises degenerate oligonucleotide libraries. This method allows the subsequent analysis of the encoding nucleic acid and thus gives 10 direct sequence information for the mimotope (see for example, Cull *et al.* (1992); Matteakis *et al.*, (1994)).

Phage display technology also provides a vehicle that allows for the selection of displayed peptides, oligopeptides or polypeptides and that simultaneously provides a link between phenotype and genotype so that the encoding nucleic acid can be identified and analysed 15 (for a review see Clackson and Wells (1994) *Trends Biotechnol* 12: 173-184). Filamentous phage particles act as genetic display packages with proteins on the outside and the nucleic acids that encode them on the inside. The practical limit on library size allowed by this technology is of the order of 10^7 to 10^{11} variants, so allowing the generation of a huge number of different compounds. This technology also allows iterative rounds of selection 20 to be performed, so honing the affinity of the molecules isolated.

The preferred method of generation of peptide, oligopeptide or polypeptide compounds that are functional equivalents of the proteins of the invention is through selection of candidate compounds in a phage display library.

Selection of a nucleic acid or gene from a phage display library will in most cases require 25 the screening of a large number of variant nucleic acids or genes. Libraries of nucleic acids or genes for use with phage display technology may be generated in a variety of ways. For example, pools of naturally-occurring genes may be cloned from genomic DNA or cDNA (see Sambrook *et al.*, 1989). Phage-antibody libraries, made by PCR amplification repertoires of antibody genes from immunised or non-immunised donors have proved very 30 effective sources of functional antibody fragments (Winter *et al.*, (1994) *Annu Rev Immunol*, 12: 433-55; Hoogenboom, (1997) *Trends Biotechnol.* 15: 62-70).

Libraries of genes can also be made by encoding all or part of genes or pools of genes or by using randomised or doped synthetic oligonucleotides. Libraries can also be made by randomly introducing mutations into a gene or into a pool of genes by a variety of techniques *in vivo*, including using so-called 'mutator strains' of bacteria such as *E. coli mutD5* (Liao *et al.*, (1986) *P.N.A.S.USA*, **83**: 576-580).

Random mutations can also be introduced both *in vivo* and *in vitro* by chemical mutagens, and ionizing or UV irradiation (see Friedberg *et al.*, (1995) *DNA repair and mutagenesis*. ASM Press, Washington), or by incorporation of mutagenic base analogues (Zaccollo *et al.*, (1996) *J Mol Biol* **255**: 589-603). Mutations can also be introduced into genes *in vitro* during 10 polymerisation, for example by using error-prone polymerases (Leung *et al.*, (1989) *Technique*, **1**: 11-15). Further diversification can be introduced by using homologous recombination either *in vivo* (see Kowalczykowski *et al.*, (1994) *Microbiol Rev*, **58**: 401-465) or *in vitro* (Stemmer, (1994) *Nature*, **370**: 389-391). Alternatively, directed mutagenesis 15 may be performed according to methods well known in the art (see McPherson *et al.*, (1991) *Directed mutagenesis. A Practical Approach*. IRL Press, Oxford).

The proteins may be specific for a class of VEGF receptors for example, the proteins may bind with high affinity to both the FLT-1, KDR/FLK and FLT-4 VEGF receptors, thus abolishing VEGF-mediated activity via all these receptor types.

In an alternative embodiment, the proteins of the invention may be specific for one 20 particular type of receptor, for example, FLT-1. In this manner, specific VEGF pathways can be targeted, that are responsible for a particular disease, or for a particular aspect of a disease. In this manner, the risk of toxic side-effects can be reduced.

In a further embodiment, the proteins of the invention may comprise hybrid molecules 25 consisting of multiple components. Such proteins of the invention may consist of repeated amino acid sequences of the fourth Ig-like domain of one or more VEGF receptors. Proteins of this aspect of the invention may dimerise more efficiently to VEGF receptor target molecules through possessing multiple binding sites.

In an alternative embodiment, such repeated domains may be derived from different receptors. For example, a protein may comprise an amino acid sequence derived partially 30 from the fourth Ig-like domain of the FLT-1 receptor and partially from the fourth Ig-like domain of the KDR/FLK or FLT-4 receptor. In this manner, proteins according to the

invention may be designed rationally so as to impart specific binding properties of interest, such as increased affinity for a certain receptor molecule. The protein may thus be designed so as to interfere with a specific dimerisation event, for example, the dimerisation of the FLT-1 receptor. The normal dimerisation process exhibited by other VEGF receptors, for example a KDR receptor, may therefore be unaffected.

According to a further aspect of the invention there is provided a protein or functional equivalent according to any one of the above-described aspects of the invention that has been genetically or chemically fused to one or more peptides or polypeptides. For example, dimerisation of the proteins of the invention with each other might be prevented by fusing

10 the fourth Ig-like domain to an effector domain that acts in the absence of ligand to prevent homodimerisation of the protein. On binding of a ligand to the effector domain, a change in the conformation of the fusion protein would allow dissociation of the fourth Ig-like domain portion of the protein, leaving it free to bind to its target VEGF receptor molecule *in situ*.

15 Other components suitable for fusion with proteins of the invention include labels, such as radioactive, enzymatic, fluorescent, or antibody label. In this embodiment of the invention, fusion proteins can be used as diagnostic tools in the evaluation of the disease state of a patient. Other suitable components for fusion include bioactive moieties such as toxins that could be delivered to specific cell types.

20 The proteins of the invention are preferably recombinant, meaning that they are derived by recombinant DNA technology. Recombinant expression of proteins allows a high level of expression to be obtained at an economic cost. Recombinant expression is widely known in the art and involves the incorporation of the gene encoding the protein of interest into an expression vector. Such an expression vector will incorporate appropriate transcriptional

25 and translational control sequences, for example enhancer elements, promoter-operator regions, termination stop sequences, mRNA stability sequences, start and stop codons or ribosomal binding sites, linked in-frame with the gene encoding the protein of interest. Secretion signalling and processing sequences may also be appropriate. Many suitable vectors and expression systems are well known and documented in the art (see, for

30 example, Sambrook *et al.*, Molecular Cloning: a laboratory manual; Cold Spring Harbor Laboratory Press; Fernandez & Hoeffler, 1998). Particularly suitable viral vectors include baculovirus-, adenovirus- and vaccinia virus-based vectors.

The proteins of the invention may be expressed recombinantly in prokaryotic hosts, such as in *E. coli*, or in eukaryotic yeasts that can be made to express high levels of recombinant protein and that can be grown easily in large quantities. Mammalian cell lines grown *in vitro* are also suitable, particularly when using virus-driven expression systems. Another 5 suitable expression system is the baculovirus expression system that involves the use of insect cells as hosts. An expression system may also constitute host cells that have the encoding DNA incorporated into their genome. Recombinant protein may easily be purified from these host cells in large quantities and at an economic cost.

Proteins for the treatment of diseases such as cancer or endometriosis will generally be 10 administered to patients as pharmaceutical compositions in therapeutically-effective doses. The term "therapeutically effective dose" as used herein refers to an amount of a therapeutic agent that is effective to treat, ameliorate, or prevent the disease in question, or to exhibit a detectable therapeutic or preventative effect. The precise effective amount for a subject will depend upon the subject's size and health, the nature and extent of the disease 15 condition, and the therapeutic agent or combination of therapeutic agents selected for administration. The effective dose for each given situation can be determined by routine experimentation and is within the judgement of the skilled person. For example, in order to formulate a range of dosage values, cell culture assays and animal studies can be used.

The dosage of such compounds preferably lies within the dose that is therapeutically 20 effective, and that exhibits little or no toxicity at this level. For example, an effective parenteral dose will be between 0.01 mg/ kg and 50 mg/kg or, more typically, between 0.05 mg/kg and 10 mg/kg of the individual to which it is administered.

According to a further aspect of the invention there is provided a protein according to any one of the above-described aspects of the invention, for use as a pharmaceutical.

25 The invention also provides the use of a protein according to any one of the above-described aspects of the invention in the manufacture of a medicament for the treatment of cancer, endometriosis, inflammation, psoriasis, rheumatoid arthritis, hemangiomas, diabetic retinopathy, angiofibromas, macular degeneration, retinal neovascularisation or any other disorder whose pathology is dependent upon a VEGF family-mediated pathway.

30 Specific neoplasms and neoplastic conditions that are amenable to treatment include breast carcinomas, lung carcinomas, gastric carcinomas, oesophageal carcinomas, colorectal

carcinomas, liver carcinomas, ovarian carcinomas, thecomas, arrhenoblastomas, cervical carcinomas, endometrial carcinomas, endometrial hyperplasias, fibrosarcomas, choriocarcinomas, head and neck cancers, nasopharyngeal carcinomas, hemangiomas, laryngeal carcinomas, hepatoblastomas, Kaposi's sarcomas, melanomas, skin carcinomas, 5 cavernous hemangiomas, hemangioblastomas, pancreas carcinomas, retinoblastomas, astrocytomas, glioblastomas, Schwannomas, oligodendrogiomas, medulloblastomas, neurblastomas, rhabdomyosarcomas, osteogenic sarcomas, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumour, renal cell carcinomas, prostate carcinomas, abnormal vascular proliferation associated with phakomatoses, oedemas (such 10 as that associated with brain tumours) and Meig's syndrome.

Specific non-neoplastic conditions that are amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other retinopathies, retrothalamic fibroplasias, leiomyomas, neovascular glaucomas, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, 15 nephrotic syndrome, pre-eclampsia, ascites, pericardial effusion (such as that associated with pericarditis) and pleural effusion.

The binding of proteins of the invention, or functional equivalents thereof, to the full length VEGF receptor or to truncated forms thereof can be used in high throughput screens for the identification of small molecular weight drug substances, such as small natural or 20 synthetic organic molecules of up to 2000Da, preferably 800Da or less in size that are effective in inhibiting dimerisation of a VEGF receptor. These compounds may be peptidic or non-peptidic in nature. The assays can be radioactive, fluorescent, colorimetric, enzymatic, chemiluminescent or any other assay type that allows the quantitation of bound substance. In an alternative to the use of binding assays, enzyme assays such as tyrosine 25 kinase assays may be used to assess the degree to which signal transduction is inhibited.

According to a still further aspect of the invention there is provided a nucleic acid encoding a protein according to any one of the above-described aspects of the invention. Such nucleic acid molecules may be incorporated into a suitable vector, which itself may be used to transfect a suitable host cell.

30 Nucleic acid molecules according to this aspect of the invention may in one aspect be used in the recombinant expression of the proteins of the above-described aspects of the invention.

In another aspect, such nucleic acid molecules may be used in gene therapy, to effect expression of the protein *in situ*. Gene therapy vehicles may comprise non-viral agents such as liposome formulations or may comprise recombinant viral vectors. Suitable viral vectors include, for example, vectors derived from retroviruses, adenoviruses, adeno-
5 associated viruses, herpes viruses or papilloma viruses. Non-viral vectors include simple plasmids formulated, for example, as liposomes (Templeton and Lasic, (1999) Mol Biotechnol 11(2):175-80). Expression of the coding sequence can be induced using endogenous mammalian or heterologous promoters, and may be either constitutive or regulated. Suitable techniques for the introduction of gene therapy vehicles into cells
10 include electroporation, the use of DNA guns, the direct injection of pure nucleic acid into tissue and liposome-mediated techniques (Dachs *et al.*, (1997) Oncol. Res. 9(6-7): 313-325; Templeton and Lasic, (1999) Mol Biotechnol 11(2): 175-80). Gene therapy vehicles may be administered either locally or systemically.

An alternative form of gene therapy involves the introduction of cells, preferably
15 autologous host cells, that contain nucleic acid sequences according to the above-described aspects of the invention into a patient suffering from a VEGF-mediated disease or disorder. The cells may comprise autologous cells harvested from the patient and transfected *ex vivo* with one or more nucleic acids encoding proteins according to the above-described aspects of the invention. These cells may then be transplanted back into the patient in areas that
20 allow for the amelioration of disease symptoms to restore the healthy function of the tissue and prevent disease progression (Bailey C.J. *et al.*, (1999) J. Mol. Med. 77(1): 244-249; Falqui L. *et al.*, (1999) Hum. Gene. Ther. 10(11): 1753-1762).

According to a further aspect of the invention there is provided a pharmaceutical composition comprising a protein or a nucleic acid according to any of the above-described
25 aspects of the invention, in conjunction with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" includes large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers and inactive virus particles. Pharmaceutically acceptable salts may also be used, for example, mineral acid salts such as
30 hydrochlorides, hydrobromides, phosphates, and sulphates, or salts of organic acids such as acetates, propionates, malonates, benzoates (see Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991)). Pharmaceutically acceptable carriers in therapeutic

compositions may also contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents and pH buffering substances, may be present.

Typically, pharmaceutical compositions are prepared as injectables, either as liquid 5 solutions or suspensions, or for application in patches. Solid forms suitable for solution in, or suspension in liquid vehicles prior to injection may also be prepared. Preparations for oral administration may also be formulated to allow for controlled release of the active compound. For administration by inhalation, the compounds of the invention may be delivered by any means known to those of the skill in the art, such as using aerosol sprays, 10 capsules or cartridges.

According to a further aspect of the invention there is provided a method for treating a patient suffering from a disorder whose pathology is dependent upon a VEGF-mediated metabolic pathway comprising administering to a patient a therapeutically-effective amount of a protein, a nucleic acid encoding such a protein, or a pharmaceutical 15 composition according to any one of the above-described aspects of the invention.

According to a still further aspect of the invention there is provided a genetically-modified animal, such as a transgenic animal, particularly a transgenic rodent animal, which expresses a protein according to any one of the above-described aspects of the invention. Methods for the production of genetically-modified animals are known in the art and 20 include techniques such as modification of somatic cells, or germ line therapy, to incorporate heritable modifications (see, for example, Rajewsky *et al.*, (1996), *J Clin Invest* 98, 600-3; Metzger and Feil, (1999) *Curr. Opinions Biotechnology* 10, 470-476; Bedell *et al.* (1997), *Genes Dev.* 11: 11-43; Bedell *et al* (1997), *Genes Dev.* 11: 1-10; "Transgenic Mammals", John Bishop (1999) Pearson Education Ltd., Harlow, Essex, for 25 example, p228). Preferably, transgenic organisms are created using germ line gene therapy.

According to a still further embodiment of the invention, there is provided a method for inhibiting the dimerisation of a VEGF receptor, comprising bringing the receptor into contact with a protein, or functional equivalent according to any one of the embodiments of the invention described above.

Various aspects and embodiments of the present invention will now be described in more detail by way of example. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

BRIEF DESCRIPTION OF THE FIGURES

5 Figure 1 gives the sequence of the *Homo sapiens* FLT-1 gene (acc. no. NM_002019)

Figure 2 gives the sequence of the *Mus musculus* FLK-1 gene (acc. no. X70842).

Figure 3 gives the sequence of the *Homo sapiens* KDR/flk-1 gene (acc. no. AF035121).

Figure 4 gives the sequence of the *Mus musculus* Flt-4 gene (acc. no. NM_008029).

Figure 5 shows an alignment of the most relevant parts of the fourth Ig-like domains of the

10 FLT-1, FLK, KDR and FLT-4 protein sequences. Residues 316-447 of domain IV of FLT-1 are shown aligned to residues 312-438 of KDR, 314-440 of FLK and 315-437 of FLT-4.

Figure 6 presents a Coomassie-stained SDS-PAGE gel showing the purification of His-Tagged Domain 4 (Construct 0) from *E. coli* crude soluble extract. Lane 1 shows unbound

15 crude protein from His-tag column after incubation with 2M Urea buffer; Lane 2 shows the bound protein after elution with 400 mM imidazole; Lane 3 shows unbound crude protein from His-tag column after incubation with 4 M Urea buffer; Lane 4 shows the bound protein after elution with 400 mM imidazole; Lane 5 shows unbound crude protein from the His-tag column after incubation with 6 M Urea buffer; and Lane 6 shows the bound

20 protein after elution with 400 mM imidazole.

Figure 7 presents a Coomassie-stained SDS-PAGE gel showing all four constructs of Domain 4 after His-tag purification. Lanes 1 to 4 show the step-wise purification of construct 0 (14.16 kDa) with increasing concentrations of imidazole to a final elution concentration of 400 mM imidazole. Lane 1 shows unbound crude protein from construct

25 0; lane 2 shows eluant after 50 mM imidazole wash; lane 3 shows eluant after 100 mM imidazole wash; and lane 4 shows eluant after 400mM imidazole wash. Lanes 5 and 6 show purification of construct 1 (13.8 kDa) after a final wash step with 100 mM imidazole (lane 5) and elution with 400 mM imidazole (lane 6). Lanes 7 and 8 show purification of construct 2 (15.12 kDa) after a final wash step with 100 mM imidazole (lane 7) and elution

30 with 400 mM imidazole (lane 8) and finally lanes 9 and 10 show purification of construct 3

(12.84 kDa) after a final wash step with 100 mM imidazole (lane 9) and elution with 400 mM imidazole (lane 10).

Figure 8 shows the specific detection of the four Domain 4 constructs by Western blotting using an anti-sFLT-1 polyclonal antibody. Lanes 1 and 2 show construct 0, lanes 3 and 4 construct 1, lanes 5 and 6 construct 2 and lanes 7 and 8 construct 3.

Figure 9 shows the specific detection of the four Domain 4 constructs by Western blotting using an anti-penta Histidine monoclonal antibody. Lane 1 shows construct 0, lane 2 construct 1, lane 3 construct 2 and lane 4 construct 3.

Figure 10 presents BIACORE data of the binding of sFLT-1 in the presence and absence of 10 all four Domain 4 constructs.

Figure 11 presents BIACORE data of the binding of all four Domain 4 constructs on the chip.

Figure 12 shows slot blot data of Domain 4 constructs 0, 1 and 2 screened with hybridomas 1H11, 6C5, 18G12, and 15F8.

15 Table 1 shows the boundaries of preferred constructs for the FLT-1, KDR, FLK and FLT-4 VEGF receptors.

Table 2 demonstrates the percentage of inhibition achieved by all four Domain 4 constructs on the binding of the sFLT-1 molecule on to the 2B2 antibody

EXAMPLES**Example 1: Preparation of FLT-1 Domain 4 molecules**

Construct 0

338 HRKQQVLETVAGKRSYRLSMKVKAFPSPEVVWLKDGLPATEKSARYLTRGYSL
 5 IIKDVTEEDAGNYTILLSIKQSNVFKNLTATLIVNVKPQIYEKAVSSFPD 440

Construct 1

330 DKAFITVKHRKQQVLETVAGKRSYRLSMKVKAFPSPEVVWLKDGLPATEK
SARYLTRGYSLIIKDVTEEDAGNYTILLSIKQSNVFKNLTATLIVNVKPQ 429

Construct 2

10 330 DKAFITVKHRKQQVLETVAGKRSYRLSMKVKAFPSPEVVWLKDGLPATEK
SARYLTRGYSLIIKDVTEEDAGNYTILLSIKQSNVFKNLTATLIVNVKPQIYEKAV
SSFPD 440

Construct 3

338 HRKQQVLETVAGKRSYRLSMKVKAFPSPEVVWLKDGLPATEKSARYLTRG
 15 YSLIIKDVTEEDAGNYTILLSIKQSNVFKNLTATLIVNVKPQ 429

The protein sequences of four potential Domain 4 constructs of FLT-1 are given above and contain residues 338-440 (Construct 0), residues 330-429 (Construct 1), 330-440 (Construct 2) and 338-429 (Construct 3) respectively. The underlined residues are predicted to be strands by 20 homology alignments to the known structures of telokin (1TLK) and domain 2 of FLT-1 (1FLT).

Any of the above constructs of the fourth Ig-like domain may be cloned by polymerase chain reaction (PCR) from the full length sFLT-1 clone using the upstream primer

Construct 0

25 (D4for) Sense 5' GGGAATTCCATATGCATCGAAAACAGCAGGTGCTTGAAAC 3',
 containing an *Nde*I site and the downstream primer
 (D4rev) Antisense 5' CGCGGATCCTTAGTCTGGAAACGATGACACGGC 3'

containing an artificial stop codon and a BamHI restriction site.

Construct 1

(Flt 39) Sense 5'CCGGTATCCATATGGATAAAGCATTCATCACTGTG3'

containing an NdeI site and the downstream primer

5 (Flt 40) Antisense 5'CGCGGGATCCTTACTGGGGTTTCACATTGACAATTAGAG 3'
containing an artificial stop codon and a BamHI restriction site.

Construct 2

(Flt 39) Sense 5'CCGGTATCCATATGGATAAAGCATTCATCACTGTG3'

containing an NdeI site and the downstream primer

10 (D4rev) Antisense 5'CGCGGGATCCTTAGTCTGGAAACGATGACACGGC3'
containing an artificial stop codon and BamHI restriction site.

Construct 3

(D4for) Sense 5'GGGAATTCCATATGCATCGAAAACAGCAGGTGCTTGAAAC3'

containing an NdeI site and the downstream primer

15 (Flt 40) Antisense 5'CGCGGGATCCTTACTGGGGTTTCACATTGACAATTAGAG 3'
containing an artificial stop codon and a BamHI restriction site. The regions
complementary to the FLT-1 molecule are shown in bold.

PCR products are purified with the QIAquick gel extraction kit (QIAGEN) and digested with *BamHI* and *NdeI*. The digested fragments are purified with the QIAquick PCR

20 purification kit (Qiagen) and ligated into the *E.coli* vector pEE14b (Novagen) that was digested by *NdeI* and *BamHI*, followed by dephosphorylation using calf intestinal alkaline phosphatase. The presence of the insert was verified by double digestion with *NdeI* and *BamHI*.

For expression, the plasmid construct is transformed in *E. coli* strain BL21(DE3) carrying
25 the inducible T7 polymerase gene. Bacterial cultures of 1lt of LB medium containing
200 μ g/ml ampicillin are grown in shaking flasks at 37°C up to an optical density of 0.7 at
600nm. The culture is grown for another 4h after addition of 50 μ M isopropyl- β -D-
thiogalactosidase at 25°C. Cells are harvested and the pellets frozen at -80°C.

Cells are lysed by mild sonication in 50mM Tris-HCl, 300mM NaCl, 10% glycerol, 0.25mM PMSF pH 8.0. Any insoluble material is removed by centrifugation at 20000g for 30min at 4°C and the histidine-tagged protein is batch absorbed onto Ni-NTA-agarose (Qiagen). The resin is washed twice with sonication buffer, followed by two washes with

5 50mM Tris-HCl, 300mM NaCl, 5mM imidazole, 20% glycerol pH8.0. The protein is eluted from the resin with 50mM Tris-HCl, 100mM NaCl, 10% glycerol, 300mM imidazole pH8.0.

Removal of the His-Tag is performed using 6 units of thrombin at 4°C overnight. The untagged protein is subsequently purified from other minor contaminants on a Superdex S-

10 100 gel-filtration column equilibrated in 20mM Tris-HCl, 150mM NaCl, pH8.0. The identity of the material is confirmed by N-terminal sequencing.

Alternative method for expression of FLT-1 Domain 4 molecules

For expression, the plasmid construct was transformed in *E. coli* strain BL21(DE3)pLysS carrying the inducible T7 RNA polymerase gene and the cells were plated onto LB agar

15 containing 50µg/ml ampicillin, 34 µg/ml chloramphenicol and 1% glucose and incubated overnight at 37°C. A single colony of these cells are aseptically picked from the agar plate and inoculated into 5 ml LB broth containing 50 µg/ml ampicillin, 34 µg/ml chloramphenicol and 1 % glucose and grown with shaking (220 rpm) overnight at 37 °C. The following morning, 1 ml of the overnight suspension is used to inoculate fresh 100 ml

20 LB broth containing 50 µg/ml ampicillin, 34 µg/ml chloramphenicol and 1 % glucose and grown with shaking at 37 °C until an optical density of 0.7 at 600 nm is reached. The cells are then pelleted by centrifugation at 3500 rpm for 15 minutes at 4 °C and resuspended in fresh 100 ml LB media containing 50 µg/ml ampicillin, 34 µg/ml chloramphenicol 1 % glucose and 0.1 mM isopropyl-β-D-thiogalactosidase in order to induce protein expression.

25 Recombinant protein is produced from the cells for 16 hours at 16 °C. The cells are harvested and the pellets frozen at - 80 °C.

Cells lysis was performed by using Novagen's Bug Buster™ Protein Extraction Reagent following the manufacturer's instructions and both the soluble clarified protein as well as inclusion bodies are collected.

30 The crude soluble fraction containing the histidine-tagged Domain 4 variants was purified using an Ni-NTA agarose column (QIAGEN). Purification was performed under

denaturing conditions, since the His-Tag tends to interact with the protein backbone, so making the protein inaccessible to the resin. Batch purification was performed by incubating the crude material overnight in buffer containing 6 M Urea, 10 mM Tris-HCl, 100 mM NaH₂PO₄ pH 8.0 with pre-equilibrated Ni-NTA resin at room temperature. The 5 resin was removed by centrifugation and the unbound supernatant removed. The Ni-NTA agarose containing absorbed His-Tagged Domain 4 protein was resuspended in wash buffer containing 6M Urea, 10 mM Tris-HCl, 100 mM NaH₂PO₄, pH8.0 and loaded onto a column fitted with a 3μm filter. The column containing resin was step-wise washed with wash buffer containing increasing concentrations of imidazole (from 25 to 100 mM) and 10 the fusion protein eluted from the resin with wash buffer containing 400 mM imidazole, pH 8.0. The identity of the eluted Domain 4 fusion proteins was confirmed by SDS-Coomassie staining and Western blotting using both an anti-His_Tag monoclonal antibody as well as an anti-Flt polyclonal antibody.

Example 2: Preliminary Analysis of Domain 4 variants

15 • **SDS Page analysis of the four Domain 4 constructs**

15 μl aliquots of protein samples were added to 3 μl of 4 x NuPAGE LDS loading buffer (Novex) and 2 μl of 10 x NuPAGE sample reducing agent (Novex), and heated at 99 °C for 5 minutes prior to loading on a 4-12 % Bis-Tris SDS PAGE gel in NuPAGE MES SDS running buffer (Novex). Protein separation was carried out for 45 minutes at 200 V. 20 Proteins were stained with Coomassie blue stain for 45 minutes and destained for 1 hour with 10 % glacial acetic acid and 30 % methanol. The results are shown in Figures 6 and 7.

• **Western blotting analysis of the four Domain 4 constructs**

Western blotting of all four constructs was performed by using anti-sFLT-1 specific 25 antibody as follows;

After electrophoresis, gels were transferred to polyvinylidene difluoride (PVDF) membrane in NuPAGE transfer buffer (Novex) with antioxidant and 20 % methanol. The transferred membranes were blocked in phosphate buffered saline (PBS) with 1 % BSA for 1 hour at room temperature prior to incubation for 1 hour in PBS with 1 % bovine gamma 30 globulin, 0.05 % Tween-20 and polyclonal goat anti-sFLT-1 biotinylated antibody (0.2ug/ml). The membranes were then washed for 5 minutes in PBS and three times in

PBS- 0.05 % Tween for 5 minutes, and finally washed for another 5 minutes in PBS. Thereafter the membranes were incubated in PBS with 1 % bovine gamma globulin, 0.05 % Tween-20 and polyclonal goat anti-biotin horseradish peroxidase (HRP) labelled antibody (dilution 1/1000) for 1 hour before thoroughly washing as previously described.

5 The membranes were then developed using Super Signal West Pico chemiluminescence substrate luminol and enhancer (Pierce) for 5 minutes prior to chemiluminescence detection reaction and exposure to X-ray film. The data are shown in Figure 8.

Additionally, Western blots of all four constructs was performed using an anti-Penta Histidine monoclonal antibody according to the manufacturer's instructions for the 10 antibody (QIAGEN). The results are shown in Figure 9.

▪ **Refolding of the four Domain 4 fusion protein constructs**

Refolding of the four constructs is necessary as they are purified under denaturing conditions. Refolding was performed by dialysis. The total protein concentration of the purified denatured protein was determined by BioRad protein assay and thereafter dialysed 15 against a 50 x volume of dialysis buffer containing 20 mM Tris-HCl, pH 7.4 at 4°C for 4 hours with constant rotation. Thereafter the buffer was changed and the fusion protein dialysed overnight at 4 °C. The protein concentration after refolding was measured by the BioRad protein assay according to manufacturer's instructions. Refolding was confirmed by Circular Dichroism (CD) analysis (data not shown).

20 All four Domain 4 variants were particularly hydrophobic and tended to fall out of solution during prolonged storage at 4°C. Out of all four constructs, constructs 1 and 2 were the least problematic, as they remained in solution even at high concentrations for few days. Construct 0 tended to precipitate very soon after refolding and concentration while construct 3 was not reliably expressed at all times.

25 The hydrophobic properties of the *E.coli*-produced Domain 4 variants may be due to lack of glycosylation. Peptide mapping experiments of sFLT-1 performed herein were consistent with one of the two potential glycosylation sites being glycosylated within Domain 4. Domain 4 contains two consensus N-glycosylation sites, 402NYT404 and 417NLT419; the peptide maps were consistent with 417NLT419 being consistently 30 glycosylated. Interestingly, this site lies within a particularly hydrophobic region of Domain 4.

Example 3: Analysis of FLT-1 Domain 4 molecules

Binding of Domain 4 on sFLT-1 is determined using Biomolecular Interaction Analysis (Biacore). (Fisher R.J. and Fivash, M. (1994) Curr. Opin. Biotech. 5, 389-395).

A monoclonal antibody (2B2) specific to Domains 1 to 3 of sFLT-1, but not Domain 4, 5 was immobilized on the sensor chip at a concentration of 30 μ g/ml in 10 mM NaAc, pH5. 50 μ g/ml of the four Domain 4 constructs in 20 mM Tris-HCl, pH7.4 were individually pre-incubated overnight at 4 °C with 50 nM sFLT-1 in HEPES buffer (0.01M Hepes, pH7.4, 0.15M NaCl, 3 mM EDTA, 0.005 % polysorbate 20 (v/v)). Thereafter the pre-incubated suspensions were passed across the immobilized monoclonal antibody and binding 10 monitored by mass sensitive detection, measured in response units (RU). A control of 50nM sFLT-1 pre-incubated overnight in HEPES buffer containing no Domain 4 was also passed across the sensor chip and its binding affinity determined. A further control was also run whereby the four Domain 4 constructs, which have not been pre-incubated with sFLT-1, were passed over the immobilized monoclonal antibody. After each run the 15 sensor chip was regenerated in 10mM Glycine pH1.75 buffer ready for the next experiment.

Figures 10 and 11 illustrate the results obtained for the four Domain 4 constructs pre-incubated with sFLT-1 prior to passing over the immobilized monoclonal antibody. In conclusion, the BIACore data have shown that pre-incubation of sFLT-1 with either of 20 Domain 4 constructs 1, 2 or 3 result in inhibition of sFLT-1 binding to the sensor chip (Table 1). In contrast, pre-incubation with construct 0 showed very poor inhibition (Table 1). These results clearly indicate that constructs 1, 2 and 3 interact with sFLT-1, preventing its interaction with the monoclonal antibody immobilized on the sensor chip. This 25 interaction is a specific domain 4-sFLT-1 interaction and is not due to direct binding competition of the Domain 4 construct to the anti-body immobilised on the chip. The data in Figure 11 have confirmed that none of the constructs bind directly onto the chip.

Alternatively, the FLT-1 molecule may be immobilised on the sensor chip and the binding affinity determined by measuring the response at the course of time at increasing concentrations of the Domain 4 protein in the presence and absence of VEGF.

30 The dimeric/monomeric state of Domain 4 as well as its interactions with sFLT-1 in solution may also be determined by gel filtration where molecules are fractionated by size.

High resolution gel filtration is used for separating the monomers from the dimers and heterodimers. Suitable columns are Sephadryl S-100 HR and S-200 HR (Pharmacia Biotech).

Direct binding of Domain 4 on the full-length receptor on the cell surface may be 5 determined by receptor-binding experiments, where radioactively or fluorescent-labelled Domain 4 binds on HUVEC cells grown to confluence in 24-well tissue culture plates under the appropriate culture conditions. For competition binding studies, labelled Domain 4 may be mixed with various concentrations of unlabelled sFLT-1. After a few hours' 10 incubation on ice, cells are washed 4 times with medium. Bound labelled Domain 4 is removed from cells by lysis with 0.1% SDS, and counts are measured in a γ counter.

The HUVEC proliferation assay (Clauss *et al.*, 1996, *J. Biol. Chem.* 271 (3): 17629-17634) may be also used to determine the inhibitory effect of the Domain 4 protein molecules on receptor dimerisation and subsequent cell proliferation. HUVECs are seeded in growth medium (EBM containing 2%FBS and GA-1000) at 5000 cells/well in 96-well cluster 15 plates. The plates are left overnight for cell attachment/stabilisation and the cells are then treated with VEGF in the presence and absence of Domain 4 molecules at variable concentrations. Cell proliferation is measured 48hrs after incubation by the Br-dU ELISA (Boehringer Mannheim) (Porstmann *et al.*, (1985) *J. Immunol. Methods* 82: 169-179).

Example 4: Preparation of a monoclonal antibody against Domain 4

20 Alternatively, an antibody, preferably a monoclonal antibody, recognising Domain 4 may be used to restrict dimerisation of the receptor. One antibody recognising Domain 4 has been produced herein and the method for its production is described below.

Three hosts were immunised with sFLT-1 produced in CHO cells in Freund's complete adjuvant, intra-muscular, followed by 3 immunisations at 2 week intervals in Freund's 25 incomplete adjuvant subcutaneously, followed by a test bleed 1 week later each immunisation. A final intra-peritoneal immunisation was given 2 weeks later before spleen collection. 1 of the 3 hosts was chosen for fusion and 25 hybridomas were initially selected. 6 hybrids were selected for cloning and 3 clones from each hybridoma were transferred into larger plates and yielded sufficient cells for frozen storage.

30 The initial bleeds were screened on a 96-well plate being coated overnight with sFLT-1, 2 μ g/ml in PBS, 100 μ l/well at 4°C. The plate was blocked with 1% BSA in PBS pH 7.4

(200µl/well, 60min, 37°C). Primary incubation was performed with 50µl anti-serum diluted in assay buffer plus 50µl competing antigen (MP9814 at 5µg/ml in assay buffer) for 1hr at 37°C. Goat anti-mouse-IgG-alkaline phosphatase labelled (1:3000 dilution) was used for detection of the primary antibody bound onto the plate. The amount of labelled antibody bound was determined by an alkaline phosphatase assay, using 0.5mg/ml pNPP substrate in 9.7% diethanolamine, pH 9.6.

Hybridoma screening was performed as described for the screening of the test bleeds with the exception that undiluted hybridoma supernatant was used during the primary incubation in place of serum. Five hybridomas were positive for binding to sFLT-1, 5G10, 10 6C5, 15F8, 1H11 and 7C10 and they were screened against a number of sFLT-1 variants for binding on dot and slot blots (see Figure 12).

Dot Blot Results

Hybridoma	5G10	6C5	15F8	1H11	7C10	
Protein	conc. / neat					
sFLT-1 Dom 1-5 crude	40 µg/ml	✓	✓	low	✓	✓
sFLT-1 Dom 1-5 purified	40 µg/ml	✓	✓	low	✓	✓
sFLT-1 Dom 1-3 crude	40 µg/ml (?)	low	✗	✗	✗	low
sFLT-1 Dom1-3 partially purified	40 µg/ml (?)	low	✗	✗	✗	?
sFLT-1 Dom 1-4/5 crude	neat	✓	✓	v.v. low	✓	✓
sFLT-1 Dom 2-3	neat	✓	✗	✗	✗	v. low
sFLT-1 Dom 2 fusion	neat	✓	✗	✗	✗	✓
sFLT-1 Dom. 4 (construct 0)	neat	✗	✗	✗	✓	✗
Blank media Excel 302	neat	✗	✗	✗	✗	✗

Blank media GMEM (1% serum)	neat	×	×	×	×	×
Wild type CHO K1 media	neat	×	×	×	×	×
Mock transfected media	neat	×	×	×	×	×

All samples were loaded at volumes of 1 μ l, 5 μ l, 12.5 μ l and 25 μ l at the above concentrations, onto a nitrocellulose membrane. The membranes were blocked in a 5% skimmed milk powder solution overnight at 4°C. Primary hybridomas were prepared in

5 5% skimmed milk powder incubation buffer and secondary antibody was added at an 1:1000 dilution in 5% skimmed milk powder incubation buffer.

Hybridoma 1H11 is positive for Domain 4 (Construct 0). This hybridoma as well as hybridomas 6C5, 18G12 and 15F8 were tested for its affinity to the variants of Domain 4 by slot blots. All Domain 4 constructs tested were positive for hybridoma 1H11 while

10 hybridomas 6C5, 18G12 and 15F8 did not cross-react with any of the Domain 4 constructs and the results are shown in Figure 12.

Table 1: Preferred constructs derived from the FLT-1, FLK, KDR and FLT-4 constructs

<u>FLT-1</u>	<u>FLK</u>	<u>KDR</u>	<u>FLT-4</u>
316 to 406	314 to 404	312 to 402	315 to 403
316 to 407	314 to 405	312 to 403	315 to 404
316 to 408	314 to 406	312 to 404	315 to 405
316 to 409	314 to 407	312 to 405	315 to 406
316 to 410	314 to 408	312 to 406	315 to 407
316 to 411	314 to 409	312 to 407	315 to 408
316 to 412	314 to 410	312 to 408	315 to 409
316 to 413	314 to 411	312 to 409	315 to 410
316 to 414	314 to 412	312 to 410	315 to 411
316 to 415	314 to 413	312 to 411	315 to 412
316 to 416	314 to 414	312 to 412	315 to 413
316 to 417	314 to 415	312 to 413	315 to 414
316 to 418	314 to 416	312 to 414	315 to 415
316 to 419	314 to 417	312 to 415	315 to 416
316 to 420	314 to 418	312 to 416	315 to 417
316 to 421	314 to 419	312 to 417	315 to 418
316 to 422	314 to 420	312 to 418	315 to 419
316 to 423	314 to 421	312 to 419	315 to 420
316 to 424	314 to 422	312 to 420	315 to 421
316 to 425	314 to 423	312 to 421	315 to 422
316 to 426	314 to 424	312 to 422	315 to 423
316 to 427	314 to 425	312 to 423	315 to 424
316 to 428	314 to 426	312 to 424	315 to 425
316 to 429	314 to 427	312 to 425	315 to 426
316 to 430	314 to 428	312 to 426	315 to 427
316 to 431	314 to 429	312 to 427	315 to 428
316 to 432	314 to 430	312 to 428	315 to 429
316 to 433	314 to 431	312 to 429	315 to 430
316 to 434	314 to 432	312 to 430	315 to 431
316 to 435	314 to 433	312 to 431	315 to 432

316 to 436	314 to 434	312 to 432	315 to 433
316 to 437	314 to 435	312 to 433	315 to 434
316 to 438	314 to 436	312 to 434	315 to 435
316 to 439	314 to 437	312 to 435	315 to 436
316 to 440	314 to 438	312 to 436	315 to 437
316 to 441	314 to 439	312 to 437	316 to 403
316 to 442	314 to 440	312 to 438	316 to 404
316 to 443	315 to 404	313 to 402	316 to 405
316 to 444	315 to 405	313 to 403	316 to 406
316 to 445	315 to 406	313 to 404	316 to 407
316 to 446	315 to 407	313 to 405	316 to 408
316 to 447	315 to 408	313 to 406	316 to 409
317 to 406	315 to 409	313 to 407	316 to 410
317 to 407	315 to 410	313 to 408	316 to 411
317 to 408	315 to 411	313 to 409	316 to 412
317 to 409	315 to 412	313 to 410	316 to 413
317 to 410	315 to 413	313 to 411	316 to 414
317 to 411	315 to 414	313 to 412	316 to 415
317 to 412	315 to 415	313 to 413	316 to 416
317 to 413	315 to 416	313 to 414	316 to 417
317 to 414	315 to 417	313 to 415	316 to 418
317 to 415	315 to 418	313 to 416	316 to 419
317 to 416	315 to 419	313 to 417	316 to 420
317 to 417	315 to 420	313 to 418	316 to 421
317 to 418	315 to 421	313 to 419	316 to 422
317 to 419	315 to 422	313 to 420	316 to 423
317 to 420	315 to 423	313 to 421	316 to 424
317 to 421	315 to 424	313 to 422	316 to 425
317 to 422	315 to 425	313 to 423	316 to 426
317 to 423	315 to 426	313 to 424	316 to 427
317 to 424	315 to 427	313 to 425	316 to 428
317 to 425	315 to 428	313 to 426	316 to 429

317 to 426	315 to 429	313 to 427	316 to 430
317 to 427	315 to 430	313 to 428	316 to 431
317 to 428	315 to 431	313 to 429	316 to 432
317 to 429	315 to 432	313 to 430	316 to 433
317 to 430	315 to 433	313 to 431	316 to 434
317 to 431	315 to 434	313 to 432	316 to 435
317 to 432	315 to 435	313 to 433	316 to 436
317 to 433	315 to 436	313 to 434	316 to 437
317 to 434	315 to 437	313 to 435	317 to 403
317 to 435	315 to 438	313 to 436	317 to 404
317 to 436	315 to 439	313 to 437	317 to 405
317 to 437	315 to 440	313 to 438	317 to 406
317 to 438	316 to 404	314 to 402	317 to 407
317 to 439	316 to 405	314 to 403	317 to 408
317 to 440	316 to 406	314 to 404	317 to 409
317 to 441	316 to 407	314 to 405	317 to 410
317 to 442	316 to 408	314 to 406	317 to 411
317 to 443	316 to 409	314 to 407	317 to 412
317 to 444	316 to 410	314 to 408	317 to 413
317 to 445	316 to 411	314 to 409	317 to 414
317 to 446	316 to 412	314 to 410	317 to 415
317 to 447	316 to 413	314 to 411	317 to 416
318 to 406	316 to 414	314 to 412	317 to 417
318 to 407	316 to 415	314 to 413	317 to 418
318 to 408	316 to 416	314 to 414	317 to 419
318 to 409	316 to 417	314 to 415	317 to 420
318 to 410	316 to 418	314 to 416	317 to 421
318 to 411	316 to 419	314 to 417	317 to 422
318 to 412	316 to 420	314 to 418	317 to 423
318 to 413	316 to 421	314 to 419	317 to 424
318 to 414	316 to 422	314 to 420	317 to 425
318 to 415	316 to 423	314 to 421	317 to 426

318 to 416	316 to 424	314 to 422	317 to 427
318 to 417	316 to 425	314 to 423	317 to 428
318 to 418	316 to 426	314 to 424	317 to 429
318 to 419	316 to 427	314 to 425	317 to 430
318 to 420	316 to 428	314 to 426	317 to 431
318 to 421	316 to 429	314 to 427	317 to 432
318 to 422	316 to 430	314 to 428	317 to 433
318 to 423	316 to 431	314 to 429	317 to 434
318 to 424	316 to 432	314 to 430	317 to 435
318 to 425	316 to 433	314 to 431	317 to 436
318 to 426	316 to 434	314 to 432	317 to 437
318 to 427	316 to 435	314 to 433	318 to 403
318 to 428	316 to 436	314 to 434	318 to 404
318 to 429	316 to 437	314 to 435	318 to 405
318 to 430	316 to 438	314 to 436	318 to 406
318 to 431	316 to 439	314 to 437	318 to 407
318 to 432	316 to 440	314 to 438	318 to 408
318 to 433	317 to 404	315 to 402	318 to 409
318 to 434	317 to 405	315 to 403	318 to 410
318 to 435	317 to 406	315 to 404	318 to 411
318 to 436	317 to 407	315 to 405	318 to 412
318 to 437	317 to 408	315 to 406	318 to 413
318 to 438	317 to 409	315 to 407	318 to 414
318 to 439	317 to 410	315 to 408	318 to 415
318 to 440	317 to 411	315 to 409	318 to 416
318 to 441	317 to 412	315 to 410	318 to 417
318 to 442	317 to 413	315 to 411	318 to 418
318 to 443	317 to 414	315 to 412	318 to 419
318 to 444	317 to 415	315 to 413	318 to 420
318 to 445	317 to 416	315 to 414	318 to 421
318 to 446	317 to 417	315 to 415	318 to 422
318 to 447	317 to 418	315 to 416	318 to 423

319 to 406	317 to 419	315 to 417	318 to 424
319 to 407	317 to 420	315 to 418	318 to 425
319 to 408	317 to 421	315 to 419	318 to 426
319 to 409	317 to 422	315 to 420	318 to 427
319 to 410	317 to 423	315 to 421	318 to 428
319 to 411	317 to 424	315 to 422	318 to 429
319 to 412	317 to 425	315 to 423	318 to 430
319 to 413	317 to 426	315 to 424	318 to 431
319 to 414	317 to 427	315 to 425	318 to 432
319 to 415	317 to 428	315 to 426	318 to 433
319 to 416	317 to 429	315 to 427	318 to 434
319 to 417	317 to 430	315 to 428	318 to 435
319 to 418	317 to 431	315 to 429	318 to 436
319 to 419	317 to 432	315 to 430	318 to 437
319 to 420	317 to 433	315 to 431	319 to 403
319 to 421	317 to 434	315 to 432	319 to 404
319 to 422	317 to 435	315 to 433	319 to 405
319 to 423	317 to 436	315 to 434	319 to 406
319 to 424	317 to 437	315 to 435	319 to 407
319 to 425	317 to 438	315 to 436	319 to 408
319 to 426	317 to 439	315 to 437	319 to 409
319 to 427	317 to 440	315 to 438	319 to 410
319 to 428	318 to 404	316 to 402	319 to 411
319 to 429	318 to 405	316 to 403	319 to 412
319 to 430	318 to 406	316 to 404	319 to 413
319 to 431	318 to 407	316 to 405	319 to 414
319 to 432	318 to 408	316 to 406	319 to 415
319 to 433	318 to 409	316 to 407	319 to 416
319 to 434	318 to 410	316 to 408	319 to 417
319 to 435	318 to 411	316 to 409	319 to 418
319 to 436	318 to 412	316 to 410	319 to 419
319 to 437	318 to 413	316 to 411	319 to 420

319 to 438	318 to 414	316 to 412	319 to 421
319 to 439	318 to 415	316 to 413	319 to 422
319 to 440	318 to 416	316 to 414	319 to 423
319 to 441	318 to 417	316 to 415	319 to 424
319 to 442	318 to 418	316 to 416	319 to 425
319 to 443	318 to 419	316 to 417	319 to 426
319 to 444	318 to 420	316 to 418	319 to 427
319 to 445	318 to 421	316 to 419	319 to 428
319 to 446	318 to 422	316 to 420	319 to 429
319 to 447	318 to 423	316 to 421	319 to 430
320 to 406	318 to 424	316 to 422	319 to 431
320 to 407	318 to 425	316 to 423	319 to 432
320 to 408	318 to 426	316 to 424	319 to 433
320 to 409	318 to 427	316 to 425	319 to 434
320 to 410	318 to 428	316 to 426	319 to 435
320 to 411	318 to 429	316 to 427	319 to 436
320 to 412	318 to 430	316 to 428	319 to 437
320 to 413	318 to 431	316 to 429	320 to 403
320 to 414	318 to 432	316 to 430	320 to 404
320 to 415	318 to 433	316 to 431	320 to 405
320 to 416	318 to 434	316 to 432	320 to 406
320 to 417	318 to 435	316 to 433	320 to 407
320 to 418	318 to 436	316 to 434	320 to 408
320 to 419	318 to 437	316 to 435	320 to 409
320 to 420	318 to 438	316 to 436	320 to 410
320 to 421	318 to 439	316 to 437	320 to 411
320 to 422	318 to 440	316 to 438	320 to 412
320 to 423	319 to 404	317 to 402	320 to 413
320 to 424	319 to 405	317 to 403	320 to 414
320 to 425	319 to 406	317 to 404	320 to 415
320 to 426	319 to 407	317 to 405	320 to 416
320 to 427	319 to 408	317 to 406	320 to 417

320 to 428	319 to 409	317 to 407	320 to 418
320 to 429	319 to 410	317 to 408	320 to 419
320 to 430	319 to 411	317 to 409	320 to 420
320 to 431	319 to 412	317 to 410	320 to 421
320 to 432	319 to 413	317 to 411	320 to 422
320 to 433	319 to 414	317 to 412	320 to 423
320 to 434	319 to 415	317 to 413	320 to 424
320 to 435	319 to 416	317 to 414	320 to 425
320 to 436	319 to 417	317 to 415	320 to 426
320 to 437	319 to 418	317 to 416	320 to 427
320 to 438	319 to 419	317 to 417	320 to 428
320 to 439	319 to 420	317 to 418	320 to 429
320 to 440	319 to 421	317 to 419	320 to 430
320 to 441	319 to 422	317 to 420	320 to 431
320 to 442	319 to 423	317 to 421	320 to 432
320 to 443	319 to 424	317 to 422	320 to 433
320 to 444	319 to 425	317 to 423	320 to 434
320 to 445	319 to 426	317 to 424	320 to 435
320 to 446	319 to 427	317 to 425	320 to 436
320 to 447	319 to 428	317 to 426	320 to 437
321 to 406	319 to 429	317 to 427	321 to 403
321 to 407	319 to 430	317 to 428	321 to 404
321 to 408	319 to 431	317 to 429	321 to 405
321 to 409	319 to 432	317 to 430	321 to 406
321 to 410	319 to 433	317 to 431	321 to 407
321 to 411	319 to 434	317 to 432	321 to 408
321 to 412	319 to 435	317 to 433	321 to 409
321 to 413	319 to 436	317 to 434	321 to 410
321 to 414	319 to 437	317 to 435	321 to 411
321 to 415	319 to 438	317 to 436	321 to 412
321 to 416	319 to 439	317 to 437	321 to 413
321 to 417	319 to 440	317 to 438	321 to 414

321 to 418	320 to 404	318 to 402	321 to 415
321 to 419	320 to 405	318 to 403	321 to 416
321 to 420	320 to 406	318 to 404	321 to 417
321 to 421	320 to 407	318 to 405	321 to 418
321 to 422	320 to 408	318 to 406	321 to 419
321 to 423	320 to 409	318 to 407	321 to 420
321 to 424	320 to 410	318 to 408	321 to 421
321 to 425	320 to 411	318 to 409	321 to 422
321 to 426	320 to 412	318 to 410	321 to 423
321 to 427	320 to 413	318 to 411	321 to 424
321 to 428	320 to 414	318 to 412	321 to 425
321 to 429	320 to 415	318 to 413	321 to 426
321 to 430	320 to 416	318 to 414	321 to 427
321 to 431	320 to 417	318 to 415	321 to 428
321 to 432	320 to 418	318 to 416	321 to 429
321 to 433	320 to 419	318 to 417	321 to 430
321 to 434	320 to 420	318 to 418	321 to 431
321 to 435	320 to 421	318 to 419	321 to 432
321 to 436	320 to 422	318 to 420	321 to 433
321 to 437	320 to 423	318 to 421	321 to 434
321 to 438	320 to 424	318 to 422	321 to 435
321 to 439	320 to 425	318 to 423	321 to 436
321 to 440	320 to 426	318 to 424	321 to 437
321 to 441	320 to 427	318 to 425	322 to 403
321 to 442	320 to 428	318 to 426	322 to 404
321 to 443	320 to 429	318 to 427	322 to 405
321 to 444	320 to 430	318 to 428	322 to 406
321 to 445	320 to 431	318 to 429	322 to 407
321 to 446	320 to 432	318 to 430	322 to 408
321 to 447	320 to 433	318 to 431	322 to 409
322 to 406	320 to 434	318 to 432	322 to 410
322 to 407	320 to 435	318 to 433	322 to 411

322 to 408	320 to 436	318 to 434	322 to 412
322 to 409	320 to 437	318 to 435	322 to 413
322 to 410	320 to 438	318 to 436	322 to 414
322 to 411	320 to 439	318 to 437	322 to 415
322 to 412	320 to 440	318 to 438	322 to 416
322 to 413	321 to 404	319 to 402	322 to 417
322 to 414	321 to 405	319 to 403	322 to 418
322 to 415	321 to 406	319 to 404	322 to 419
322 to 416	321 to 407	319 to 405	322 to 420
322 to 417	321 to 408	319 to 406	322 to 421
322 to 418	321 to 409	319 to 407	322 to 422
322 to 419	321 to 410	319 to 408	322 to 423
322 to 420	321 to 411	319 to 409	322 to 424
322 to 421	321 to 412	319 to 410	322 to 425
322 to 422	321 to 413	319 to 411	322 to 426
322 to 423	321 to 414	319 to 412	322 to 427
322 to 424	321 to 415	319 to 413	322 to 428
322 to 425	321 to 416	319 to 414	322 to 429
322 to 426	321 to 417	319 to 415	322 to 430
322 to 427	321 to 418	319 to 416	322 to 431
322 to 428	321 to 419	319 to 417	322 to 432
322 to 429	321 to 420	319 to 418	322 to 433
322 to 430	321 to 421	319 to 419	322 to 434
322 to 431	321 to 422	319 to 420	322 to 435
322 to 432	321 to 423	319 to 421	322 to 436
322 to 433	321 to 424	319 to 422	322 to 437
322 to 434	321 to 425	319 to 423	323 to 403
322 to 435	321 to 426	319 to 424	323 to 404
322 to 436	321 to 427	319 to 425	323 to 405
322 to 437	321 to 428	319 to 426	323 to 406
322 to 438	321 to 429	319 to 427	323 to 407
322 to 439	321 to 430	319 to 428	323 to 408

322 to 440	321 to 431	319 to 429	323 to 409
322 to 441	321 to 432	319 to 430	323 to 410
322 to 442	321 to 433	319 to 431	323 to 411
322 to 443	321 to 434	319 to 432	323 to 412
322 to 444	321 to 435	319 to 433	323 to 413
322 to 445	321 to 436	319 to 434	323 to 414
322 to 446	321 to 437	319 to 435	323 to 415
322 to 447	321 to 438	319 to 436	323 to 416
323 to 406	321 to 439	319 to 437	323 to 417
323 to 407	321 to 440	319 to 438	323 to 418
323 to 408	322 to 404	320 to 402	323 to 419
323 to 409	322 to 405	320 to 403	323 to 420
323 to 410	322 to 406	320 to 404	323 to 421
323 to 411	322 to 407	320 to 405	323 to 422
323 to 412	322 to 408	320 to 406	323 to 423
323 to 413	322 to 409	320 to 407	323 to 424
323 to 414	322 to 410	320 to 408	323 to 425
323 to 415	322 to 411	320 to 409	323 to 426
323 to 416	322 to 412	320 to 410	323 to 427
323 to 417	322 to 413	320 to 411	323 to 428
323 to 418	322 to 414	320 to 412	323 to 429
323 to 419	322 to 415	320 to 413	323 to 430
323 to 420	322 to 416	320 to 414	323 to 431
323 to 421	322 to 417	320 to 415	323 to 432
323 to 422	322 to 418	320 to 416	323 to 433
323 to 423	322 to 419	320 to 417	323 to 434
323 to 424	322 to 420	320 to 418	323 to 435
323 to 425	322 to 421	320 to 419	323 to 436
323 to 426	322 to 422	320 to 420	323 to 437
323 to 427	322 to 423	320 to 421	324 to 403
323 to 428	322 to 424	320 to 422	324 to 404
323 to 429	322 to 425	320 to 423	324 to 405

323 to 430	322 to 426	320 to 424	324 to 406
323 to 431	322 to 427	320 to 425	324 to 407
323 to 432	322 to 428	320 to 426	324 to 408
323 to 433	322 to 429	320 to 427	324 to 409
323 to 434	322 to 430	320 to 428	324 to 410
323 to 435	322 to 431	320 to 429	324 to 411
323 to 436	322 to 432	320 to 430	324 to 412
323 to 437	322 to 433	320 to 431	324 to 413
323 to 438	322 to 434	320 to 432	324 to 414
323 to 439	322 to 435	320 to 433	324 to 415
323 to 440	322 to 436	320 to 434	324 to 416
323 to 441	322 to 437	320 to 435	324 to 417
323 to 442	322 to 438	320 to 436	324 to 418
323 to 443	322 to 439	320 to 437	324 to 419
323 to 444	322 to 440	320 to 438	324 to 420
323 to 445	323 to 404	321 to 402	324 to 421
323 to 446	323 to 405	321 to 403	324 to 422
323 to 447	323 to 406	321 to 404	324 to 423
324 to 406	323 to 407	321 to 405	324 to 424
324 to 407	323 to 408	321 to 406	324 to 425
324 to 408	323 to 409	321 to 407	324 to 426
324 to 409	323 to 410	321 to 408	324 to 427
324 to 410	323 to 411	321 to 409	324 to 428
324 to 411	323 to 412	321 to 410	324 to 429
324 to 412	323 to 413	321 to 411	324 to 430
324 to 413	323 to 414	321 to 412	324 to 431
324 to 414	323 to 415	321 to 413	324 to 432
324 to 415	323 to 416	321 to 414	324 to 433
324 to 416	323 to 417	321 to 415	324 to 434
324 to 417	323 to 418	321 to 416	324 to 435
324 to 418	323 to 419	321 to 417	324 to 436
324 to 419	323 to 420	321 to 418	324 to 437

324 to 420	323 to 421	321 to 419	325 to 403
324 to 421	323 to 422	321 to 420	325 to 404
324 to 422	323 to 423	321 to 421	325 to 405
324 to 423	323 to 424	321 to 422	325 to 406
324 to 424	323 to 425	321 to 423	325 to 407
324 to 425	323 to 426	321 to 424	325 to 408
324 to 426	323 to 427	321 to 425	325 to 409
324 to 427	323 to 428	321 to 426	325 to 410
324 to 428	323 to 429	321 to 427	325 to 411
324 to 429	323 to 430	321 to 428	325 to 412
324 to 430	323 to 431	321 to 429	325 to 413
324 to 431	323 to 432	321 to 430	325 to 414
324 to 432	323 to 433	321 to 431	325 to 415
324 to 433	323 to 434	321 to 432	325 to 416
324 to 434	323 to 435	321 to 433	325 to 417
324 to 435	323 to 436	321 to 434	325 to 418
324 to 436	323 to 437	321 to 435	325 to 419
324 to 437	323 to 438	321 to 436	325 to 420
324 to 438	323 to 439	321 to 437	325 to 421
324 to 439	323 to 440	321 to 438	325 to 422
324 to 440	324 to 404	322 to 402	325 to 423
324 to 441	324 to 405	322 to 403	325 to 424
324 to 442	324 to 406	322 to 404	325 to 425
324 to 443	324 to 407	322 to 405	325 to 426
324 to 444	324 to 408	322 to 406	325 to 427
324 to 445	324 to 409	322 to 407	325 to 428
324 to 446	324 to 410	322 to 408	325 to 429
324 to 447	324 to 411	322 to 409	325 to 430
325 to 406	324 to 412	322 to 410	325 to 431
325 to 407	324 to 413	322 to 411	325 to 432
325 to 408	324 to 414	322 to 412	325 to 433
325 to 409	324 to 415	322 to 413	325 to 434

325 to 410	324 to 416	322 to 414	325 to 435
325 to 411	324 to 417	322 to 415	325 to 436
325 to 412	324 to 418	322 to 416	325 to 437
325 to 413	324 to 419	322 to 417	326 to 403
325 to 414	324 to 420	322 to 418	326 to 404
325 to 415	324 to 421	322 to 419	326 to 405
325 to 416	324 to 422	322 to 420	326 to 406
325 to 417	324 to 423	322 to 421	326 to 407
325 to 418	324 to 424	322 to 422	326 to 408
325 to 419	324 to 425	322 to 423	326 to 409
325 to 420	324 to 426	322 to 424	326 to 410
325 to 421	324 to 427	322 to 425	326 to 411
325 to 422	324 to 428	322 to 426	326 to 412
325 to 423	324 to 429	322 to 427	326 to 413
325 to 424	324 to 430	322 to 428	326 to 414
325 to 425	324 to 431	322 to 429	326 to 415
325 to 426	324 to 432	322 to 430	326 to 416
325 to 427	324 to 433	322 to 431	326 to 417
325 to 428	324 to 434	322 to 432	326 to 418
325 to 429	324 to 435	322 to 433	326 to 419
325 to 430	324 to 436	322 to 434	326 to 420
325 to 431	324 to 437	322 to 435	326 to 421
325 to 432	324 to 438	322 to 436	326 to 422
325 to 433	324 to 439	322 to 437	326 to 423
325 to 434	324 to 440	322 to 438	326 to 424
325 to 435	325 to 404	323 to 402	326 to 425
325 to 436	325 to 405	323 to 403	326 to 426
325 to 437	325 to 406	323 to 404	326 to 427
325 to 438	325 to 407	323 to 405	326 to 428
325 to 439	325 to 408	323 to 406	326 to 429
325 to 440	325 to 409	323 to 407	326 to 430
325 to 441	325 to 410	323 to 408	326 to 431

325 to 442	325 to 411	323 to 409	326 to 432
325 to 443	325 to 412	323 to 410	326 to 433
325 to 444	325 to 413	323 to 411	326 to 434
325 to 445	325 to 414	323 to 412	326 to 435
325 to 446	325 to 415	323 to 413	326 to 436
325 to 447	325 to 416	323 to 414	326 to 437
326 to 406	325 to 417	323 to 415	327 to 403
326 to 407	325 to 418	323 to 416	327 to 404
326 to 408	325 to 419	323 to 417	327 to 405
326 to 409	325 to 420	323 to 418	327 to 406
326 to 410	325 to 421	323 to 419	327 to 407
326 to 411	325 to 422	323 to 420	327 to 408
326 to 412	325 to 423	323 to 421	327 to 409
326 to 413	325 to 424	323 to 422	327 to 410
326 to 414	325 to 425	323 to 423	327 to 411
326 to 415	325 to 426	323 to 424	327 to 412
326 to 416	325 to 427	323 to 425	327 to 413
326 to 417	325 to 428	323 to 426	327 to 414
326 to 418	325 to 429	323 to 427	327 to 415
326 to 419	325 to 430	323 to 428	327 to 416
326 to 420	325 to 431	323 to 429	327 to 417
326 to 421	325 to 432	323 to 430	327 to 418
326 to 422	325 to 433	323 to 431	327 to 419
326 to 423	325 to 434	323 to 432	327 to 420
326 to 424	325 to 435	323 to 433	327 to 421
326 to 425	325 to 436	323 to 434	327 to 422
326 to 426	325 to 437	323 to 435	327 to 423
326 to 427	325 to 438	323 to 436	327 to 424
326 to 428	325 to 439	323 to 437	327 to 425
326 to 429	325 to 440	323 to 438	327 to 426
326 to 430	326 to 404	324 to 402	327 to 427
326 to 431	326 to 405	324 to 403	327 to 428

326 to 432	326 to 406	324 to 404	327 to 429
326 to 433	326 to 407	324 to 405	327 to 430
326 to 434	326 to 408	324 to 406	327 to 431
326 to 435	326 to 409	324 to 407	327 to 432
326 to 436	326 to 410	324 to 408	327 to 433
326 to 437	326 to 411	324 to 409	327 to 434
326 to 438	326 to 412	324 to 410	327 to 435
326 to 439	326 to 413	324 to 411	327 to 436
326 to 440	326 to 414	324 to 412	327 to 437
326 to 441	326 to 415	324 to 413	328 to 403
326 to 442	326 to 416	324 to 414	328 to 404
326 to 443	326 to 417	324 to 415	328 to 405
326 to 444	326 to 418	324 to 416	328 to 406
326 to 445	326 to 419	324 to 417	328 to 407
326 to 446	326 to 420	324 to 418	328 to 408
326 to 447	326 to 421	324 to 419	328 to 409
327 to 406	326 to 422	324 to 420	328 to 410
327 to 407	326 to 423	324 to 421	328 to 411
327 to 408	326 to 424	324 to 422	328 to 412
327 to 409	326 to 425	324 to 423	328 to 413
327 to 410	326 to 426	324 to 424	328 to 414
327 to 411	326 to 427	324 to 425	328 to 415
327 to 412	326 to 428	324 to 426	328 to 416
327 to 413	326 to 429	324 to 427	328 to 417
327 to 414	326 to 430	324 to 428	328 to 418
327 to 415	326 to 431	324 to 429	328 to 419
327 to 416	326 to 432	324 to 430	328 to 420
327 to 417	326 to 433	324 to 431	328 to 421
327 to 418	326 to 434	324 to 432	328 to 422
327 to 419	326 to 435	324 to 433	328 to 423
327 to 420	326 to 436	324 to 434	328 to 424
327 to 421	326 to 437	324 to 435	328 to 425

327 to 422	326 to 438	324 to 436	328 to 426
327 to 423	326 to 439	324 to 437	328 to 427
327 to 424	326 to 440	324 to 438	328 to 428
327 to 425	327 to 404	325 to 402	328 to 429
327 to 426	327 to 405	325 to 403	328 to 430
327 to 427	327 to 406	325 to 404	328 to 431
327 to 428	327 to 407	325 to 405	328 to 432
327 to 429	327 to 408	325 to 406	328 to 433
327 to 430	327 to 409	325 to 407	328 to 434
327 to 431	327 to 410	325 to 408	328 to 435
327 to 432	327 to 411	325 to 409	328 to 436
327 to 433	327 to 412	325 to 410	328 to 437
327 to 434	327 to 413	325 to 411	329 to 403
327 to 435	327 to 414	325 to 412	329 to 404
327 to 436	327 to 415	325 to 413	329 to 405
327 to 437	327 to 416	325 to 414	329 to 406
327 to 438	327 to 417	325 to 415	329 to 407
327 to 439	327 to 418	325 to 416	329 to 408
327 to 440	327 to 419	325 to 417	329 to 409
327 to 441	327 to 420	325 to 418	329 to 410
327 to 442	327 to 421	325 to 419	329 to 411
327 to 443	327 to 422	325 to 420	329 to 412
327 to 444	327 to 423	325 to 421	329 to 413
327 to 445	327 to 424	325 to 422	329 to 414
327 to 446	327 to 425	325 to 423	329 to 415
327 to 447	327 to 426	325 to 424	329 to 416
328 to 406	327 to 427	325 to 425	329 to 417
328 to 407	327 to 428	325 to 426	329 to 418
328 to 408	327 to 429	325 to 427	329 to 419
328 to 409	327 to 430	325 to 428	329 to 420
328 to 410	327 to 431	325 to 429	329 to 421
328 to 411	327 to 432	325 to 430	329 to 422

328 to 412	327 to 433	325 to 431	329 to 423
328 to 413	327 to 434	325 to 432	329 to 424
328 to 414	327 to 435	325 to 433	329 to 425
328 to 415	327 to 436	325 to 434	329 to 426
328 to 416	327 to 437	325 to 435	329 to 427
328 to 417	327 to 438	325 to 436	329 to 428
328 to 418	327 to 439	325 to 437	329 to 429
328 to 419	327 to 440	325 to 438	329 to 430
328 to 420	328 to 404	326 to 402	329 to 431
328 to 421	328 to 405	326 to 403	329 to 432
328 to 422	328 to 406	326 to 404	329 to 433
328 to 423	328 to 407	326 to 405	329 to 434
328 to 424	328 to 408	326 to 406	329 to 435
328 to 425	328 to 409	326 to 407	329 to 436
328 to 426	328 to 410	326 to 408	329 to 437
328 to 427	328 to 411	326 to 409	330 to 403
328 to 428	328 to 412	326 to 410	330 to 404
328 to 429	328 to 413	326 to 411	330 to 405
328 to 430	328 to 414	326 to 412	330 to 406
328 to 431	328 to 415	326 to 413	330 to 407
328 to 432	328 to 416	326 to 414	330 to 408
328 to 433	328 to 417	326 to 415	330 to 409
328 to 434	328 to 418	326 to 416	330 to 410
328 to 435	328 to 419	326 to 417	330 to 411
328 to 436	328 to 420	326 to 418	330 to 412
328 to 437	328 to 421	326 to 419	330 to 413
328 to 438	328 to 422	326 to 420	330 to 414
328 to 439	328 to 423	326 to 421	330 to 415
328 to 440	328 to 424	326 to 422	330 to 416
328 to 441	328 to 425	326 to 423	330 to 417
328 to 442	328 to 426	326 to 424	330 to 418
328 to 443	328 to 427	326 to 425	330 to 419

328 to 444	328 to 428	326 to 426	330 to 420
328 to 445	328 to 429	326 to 427	330 to 421
328 to 446	328 to 430	326 to 428	330 to 422
328 to 447	328 to 431	326 to 429	330 to 423
329 to 406	328 to 432	326 to 430	330 to 424
329 to 407	328 to 433	326 to 431	330 to 425
329 to 408	328 to 434	326 to 432	330 to 426
329 to 409	328 to 435	326 to 433	330 to 427
329 to 410	328 to 436	326 to 434	330 to 428
329 to 411	328 to 437	326 to 435	330 to 429
329 to 412	328 to 438	326 to 436	330 to 430
329 to 413	328 to 439	326 to 437	330 to 431
329 to 414	328 to 440	326 to 438	330 to 432
329 to 415	329 to 404	327 to 402	330 to 433
329 to 416	329 to 405	327 to 403	330 to 434
329 to 417	329 to 406	327 to 404	330 to 435
329 to 418	329 to 407	327 to 405	330 to 436
329 to 419	329 to 408	327 to 406	330 to 437
329 to 420	329 to 409	327 to 407	331 to 403
329 to 421	329 to 410	327 to 408	331 to 404
329 to 422	329 to 411	327 to 409	331 to 405
329 to 423	329 to 412	327 to 410	331 to 406
329 to 424	329 to 413	327 to 411	331 to 407
329 to 425	329 to 414	327 to 412	331 to 408
329 to 426	329 to 415	327 to 413	331 to 409
329 to 427	329 to 416	327 to 414	331 to 410
329 to 428	329 to 417	327 to 415	331 to 411
329 to 429	329 to 418	327 to 416	331 to 412
329 to 430	329 to 419	327 to 417	331 to 413
329 to 431	329 to 420	327 to 418	331 to 414
329 to 432	329 to 421	327 to 419	331 to 415
329 to 433	329 to 422	327 to 420	331 to 416

329 to 434	329 to 423	327 to 421	331 to 417
329 to 435	329 to 424	327 to 422	331 to 418
329 to 436	329 to 425	327 to 423	331 to 419
329 to 437	329 to 426	327 to 424	331 to 420
329 to 438	329 to 427	327 to 425	331 to 421
329 to 439	329 to 428	327 to 426	331 to 422
329 to 440	329 to 429	327 to 427	331 to 423
329 to 441	329 to 430	327 to 428	331 to 424
329 to 442	329 to 431	327 to 429	331 to 425
329 to 443	329 to 432	327 to 430	331 to 426
329 to 444	329 to 433	327 to 431	331 to 427
329 to 445	329 to 434	327 to 432	331 to 428
329 to 446	329 to 435	327 to 433	331 to 429
329 to 447	329 to 436	327 to 434	331 to 430
330 to 406	329 to 437	327 to 435	331 to 431
330 to 407	329 to 438	327 to 436	331 to 432
330 to 408	329 to 439	327 to 437	331 to 433
330 to 409	329 to 440	327 to 438	331 to 434
330 to 410	330 to 404	328 to 402	331 to 435
330 to 411	330 to 405	328 to 403	331 to 436
330 to 412	330 to 406	328 to 404	331 to 437
330 to 413	330 to 407	328 to 405	332 to 403
330 to 414	330 to 408	328 to 406	332 to 404
330 to 415	330 to 409	328 to 407	332 to 405
330 to 416	330 to 410	328 to 408	332 to 406
330 to 417	330 to 411	328 to 409	332 to 407
330 to 418	330 to 412	328 to 410	332 to 408
330 to 419	330 to 413	328 to 411	332 to 409
330 to 420	330 to 414	328 to 412	332 to 410
330 to 421	330 to 415	328 to 413	332 to 411
330 to 422	330 to 416	328 to 414	332 to 412
330 to 423	330 to 417	328 to 415	332 to 413

330 to 424	330 to 418	328 to 416	332 to 414
330 to 425	330 to 419	328 to 417	332 to 415
330 to 426	330 to 420	328 to 418	332 to 416
330 to 427	330 to 421	328 to 419	332 to 417
330 to 428	330 to 422	328 to 420	332 to 418
330 to 429	330 to 423	328 to 421	332 to 419
330 to 430	330 to 424	328 to 422	332 to 420
330 to 431	330 to 425	328 to 423	332 to 421
330 to 432	330 to 426	328 to 424	332 to 422
330 to 433	330 to 427	328 to 425	332 to 423
330 to 434	330 to 428	328 to 426	332 to 424
330 to 435	330 to 429	328 to 427	332 to 425
330 to 436	330 to 430	328 to 428	332 to 426
330 to 437	330 to 431	328 to 429	332 to 427
330 to 438	330 to 432	328 to 430	332 to 428
330 to 439	330 to 433	328 to 431	332 to 429
330 to 440	330 to 434	328 to 432	332 to 430
330 to 441	330 to 435	328 to 433	332 to 431
330 to 442	330 to 436	328 to 434	332 to 432
330 to 443	330 to 437	328 to 435	332 to 433
330 to 444	330 to 438	328 to 436	332 to 434
330 to 445	330 to 439	328 to 437	332 to 435
330 to 446	330 to 440	328 to 438	332 to 436
330 to 447	331 to 404	329 to 402	332 to 437
331 to 406	331 to 405	329 to 403	333 to 403
331 to 407	331 to 406	329 to 404	333 to 404
331 to 408	331 to 407	329 to 405	333 to 405
331 to 409	331 to 408	329 to 406	333 to 406
331 to 410	331 to 409	329 to 407	333 to 407
331 to 411	331 to 410	329 to 408	333 to 408
331 to 412	331 to 411	329 to 409	333 to 409
331 to 413	331 to 412	329 to 410	333 to 410

331 to 414	331 to 413	329 to 411	333 to 411
331 to 415	331 to 414	329 to 412	333 to 412
331 to 416	331 to 415	329 to 413	333 to 413
331 to 417	331 to 416	329 to 414	333 to 414
331 to 418	331 to 417	329 to 415	333 to 415
331 to 419	331 to 418	329 to 416	333 to 416
331 to 420	331 to 419	329 to 417	333 to 417
331 to 421	331 to 420	329 to 418	333 to 418
331 to 422	331 to 421	329 to 419	333 to 419
331 to 423	331 to 422	329 to 420	333 to 420
331 to 424	331 to 423	329 to 421	333 to 421
331 to 425	331 to 424	329 to 422	333 to 422
331 to 426	331 to 425	329 to 423	333 to 423
331 to 427	331 to 426	329 to 424	333 to 424
331 to 428	331 to 427	329 to 425	333 to 425
331 to 429	331 to 428	329 to 426	333 to 426
331 to 430	331 to 429	329 to 427	333 to 427
331 to 431	331 to 430	329 to 428	333 to 428
331 to 432	331 to 431	329 to 429	333 to 429
331 to 433	331 to 432	329 to 430	333 to 430
331 to 434	331 to 433	329 to 431	333 to 431
331 to 435	331 to 434	329 to 432	333 to 432
331 to 436	331 to 435	329 to 433	333 to 433
331 to 437	331 to 436	329 to 434	333 to 434
331 to 438	331 to 437	329 to 435	333 to 435
331 to 439	331 to 438	329 to 436	333 to 436
331 to 440	331 to 439	329 to 437	333 to 437
331 to 441	331 to 440	329 to 438	334 to 403
331 to 442	332 to 404	330 to 402	334 to 404
331 to 443	332 to 405	330 to 403	334 to 405
331 to 444	332 to 406	330 to 404	334 to 406
331 to 445	332 to 407	330 to 405	334 to 407

331 to 446	332 to 408	330 to 406	334 to 408
331 to 447	332 to 409	330 to 407	334 to 409
332 to 406	332 to 410	330 to 408	334 to 410
332 to 407	332 to 411	330 to 409	334 to 411
332 to 408	332 to 412	330 to 410	334 to 412
332 to 409	332 to 413	330 to 411	334 to 413
332 to 410	332 to 414	330 to 412	334 to 414
332 to 411	332 to 415	330 to 413	334 to 415
332 to 412	332 to 416	330 to 414	334 to 416
332 to 413	332 to 417	330 to 415	334 to 417
332 to 414	332 to 418	330 to 416	334 to 418
332 to 415	332 to 419	330 to 417	334 to 419
332 to 416	332 to 420	330 to 418	334 to 420
332 to 417	332 to 421	330 to 419	334 to 421
332 to 418	332 to 422	330 to 420	334 to 422
332 to 419	332 to 423	330 to 421	334 to 423
332 to 420	332 to 424	330 to 422	334 to 424
332 to 421	332 to 425	330 to 423	334 to 425
332 to 422	332 to 426	330 to 424	334 to 426
332 to 423	332 to 427	330 to 425	334 to 427
332 to 424	332 to 428	330 to 426	334 to 428
332 to 425	332 to 429	330 to 427	334 to 429
332 to 426	332 to 430	330 to 428	334 to 430
332 to 427	332 to 431	330 to 429	334 to 431
332 to 428	332 to 432	330 to 430	334 to 432
332 to 429	332 to 433	330 to 431	334 to 433
332 to 430	332 to 434	330 to 432	334 to 434
332 to 431	332 to 435	330 to 433	334 to 435
332 to 432	332 to 436	330 to 434	334 to 436
332 to 433	332 to 437	330 to 435	334 to 437
332 to 434	332 to 438	330 to 436	335 to 403
332 to 435	332 to 439	330 to 437	335 to 404

332 to 436	332 to 440	330 to 438	335 to 405
332 to 437	333 to 404	331 to 402	335 to 406
332 to 438	333 to 405	331 to 403	335 to 407
332 to 439	333 to 406	331 to 404	335 to 408
332 to 440	333 to 407	331 to 405	335 to 409
332 to 441	333 to 408	331 to 406	335 to 410
332 to 442	333 to 409	331 to 407	335 to 411
332 to 443	333 to 410	331 to 408	335 to 412
332 to 444	333 to 411	331 to 409	335 to 413
332 to 445	333 to 412	331 to 410	335 to 414
332 to 446	333 to 413	331 to 411	335 to 415
332 to 447	333 to 414	331 to 412	335 to 416
333 to 406	333 to 415	331 to 413	335 to 417
333 to 407	333 to 416	331 to 414	335 to 418
333 to 408	333 to 417	331 to 415	335 to 419
333 to 409	333 to 418	331 to 416	335 to 420
333 to 410	333 to 419	331 to 417	335 to 421
333 to 411	333 to 420	331 to 418	335 to 422
333 to 412	333 to 421	331 to 419	335 to 423
333 to 413	333 to 422	331 to 420	335 to 424
333 to 414	333 to 423	331 to 421	335 to 425
333 to 415	333 to 424	331 to 422	335 to 426
333 to 416	333 to 425	331 to 423	335 to 427
333 to 417	333 to 426	331 to 424	335 to 428
333 to 418	333 to 427	331 to 425	335 to 429
333 to 419	333 to 428	331 to 426	335 to 430
333 to 420	333 to 429	331 to 427	335 to 431
333 to 421	333 to 430	331 to 428	335 to 432
333 to 422	333 to 431	331 to 429	335 to 433
333 to 423	333 to 432	331 to 430	335 to 434
333 to 424	333 to 433	331 to 431	335 to 435
333 to 425	333 to 434	331 to 432	335 to 436

333 to 426	333 to 435	331 to 433	335 to 437
333 to 427	333 to 436	331 to 434	336 to 403
333 to 428	333 to 437	331 to 435	336 to 404
333 to 429	333 to 438	331 to 436	336 to 405
333 to 430	333 to 439	331 to 437	336 to 406
333 to 431	333 to 440	331 to 438	336 to 407
333 to 432	334 to 404	332 to 402	336 to 408
333 to 433	334 to 405	332 to 403	336 to 409
333 to 434	334 to 406	332 to 404	336 to 410
333 to 435	334 to 407	332 to 405	336 to 411
333 to 436	334 to 408	332 to 406	336 to 412
333 to 437	334 to 409	332 to 407	336 to 413
333 to 438	334 to 410	332 to 408	336 to 414
333 to 439	334 to 411	332 to 409	336 to 415
333 to 440	334 to 412	332 to 410	336 to 416
333 to 441	334 to 413	332 to 411	336 to 417
333 to 442	334 to 414	332 to 412	336 to 418
333 to 443	334 to 415	332 to 413	336 to 419
333 to 444	334 to 416	332 to 414	336 to 420
333 to 445	334 to 417	332 to 415	336 to 421
333 to 446	334 to 418	332 to 416	336 to 422
333 to 447	334 to 419	332 to 417	336 to 423
334 to 406	334 to 420	332 to 418	336 to 424
334 to 407	334 to 421	332 to 419	336 to 425
334 to 408	334 to 422	332 to 420	336 to 426
334 to 409	334 to 423	332 to 421	336 to 427
334 to 410	334 to 424	332 to 422	336 to 428
334 to 411	334 to 425	332 to 423	336 to 429
334 to 412	334 to 426	332 to 424	336 to 430
334 to 413	334 to 427	332 to 425	336 to 431
334 to 414	334 to 428	332 to 426	336 to 432
334 to 415	334 to 429	332 to 427	336 to 433

334 to 416	334 to 430	332 to 428	336 to 434
334 to 417	334 to 431	332 to 429	336 to 435
334 to 418	334 to 432	332 to 430	336 to 436
334 to 419	334 to 433	332 to 431	336 to 437
334 to 420	334 to 434	332 to 432	337 to 403
334 to 421	334 to 435	332 to 433	337 to 404
334 to 422	334 to 436	332 to 434	337 to 405
334 to 423	334 to 437	332 to 435	337 to 406
334 to 424	334 to 438	332 to 436	337 to 407
334 to 425	334 to 439	332 to 437	337 to 408
334 to 426	334 to 440	332 to 438	337 to 409
334 to 427	335 to 404	333 to 402	337 to 410
334 to 428	335 to 405	333 to 403	337 to 411
334 to 429	335 to 406	333 to 404	337 to 412
334 to 430	335 to 407	333 to 405	337 to 413
334 to 431	335 to 408	333 to 406	337 to 414
334 to 432	335 to 409	333 to 407	337 to 415
334 to 433	335 to 410	333 to 408	337 to 416
334 to 434	335 to 411	333 to 409	337 to 417
334 to 435	335 to 412	333 to 410	337 to 418
334 to 436	335 to 413	333 to 411	337 to 419
334 to 437	335 to 414	333 to 412	337 to 420
334 to 438	335 to 415	333 to 413	337 to 421
334 to 439	335 to 416	333 to 414	337 to 422
334 to 440	335 to 417	333 to 415	337 to 423
334 to 441	335 to 418	333 to 416	337 to 424
334 to 442	335 to 419	333 to 417	337 to 425
334 to 443	335 to 420	333 to 418	337 to 426
334 to 444	335 to 421	333 to 419	337 to 427
334 to 445	335 to 422	333 to 420	337 to 428
334 to 446	335 to 423	333 to 421	337 to 429
334 to 447	335 to 424	333 to 422	337 to 430

335 to 406	335 to 425	333 to 423	337 to 431
335 to 407	335 to 426	333 to 424	337 to 432
335 to 408	335 to 427	333 to 425	337 to 433
335 to 409	335 to 428	333 to 426	337 to 434
335 to 410	335 to 429	333 to 427	337 to 435
335 to 411	335 to 430	333 to 428	337 to 436
335 to 412	335 to 431	333 to 429	337 to 437
335 to 413	335 to 432	333 to 430	338 to 403
335 to 414	335 to 433	333 to 431	338 to 404
335 to 415	335 to 434	333 to 432	338 to 405
335 to 416	335 to 435	333 to 433	338 to 406
335 to 417	335 to 436	333 to 434	338 to 407
335 to 418	335 to 437	333 to 435	338 to 408
335 to 419	335 to 438	333 to 436	338 to 409
335 to 420	335 to 439	333 to 437	338 to 410
335 to 421	335 to 440	333 to 438	338 to 411
335 to 422	336 to 404	334 to 402	338 to 412
335 to 423	336 to 405	334 to 403	338 to 413
335 to 424	336 to 406	334 to 404	338 to 414
335 to 425	336 to 407	334 to 405	338 to 415
335 to 426	336 to 408	334 to 406	338 to 416
335 to 427	336 to 409	334 to 407	338 to 417
335 to 428	336 to 410	334 to 408	338 to 418
335 to 429	336 to 411	334 to 409	338 to 419
335 to 430	336 to 412	334 to 410	338 to 420
335 to 431	336 to 413	334 to 411	338 to 421
335 to 432	336 to 414	334 to 412	338 to 422
335 to 433	336 to 415	334 to 413	338 to 423
335 to 434	336 to 416	334 to 414	338 to 424
335 to 435	336 to 417	334 to 415	338 to 425
335 to 436	336 to 418	334 to 416	338 to 426
335 to 437	336 to 419	334 to 417	338 to 427

335 to 438	336 to 420	334 to 418	338 to 428
335 to 439	336 to 421	334 to 419	338 to 429
335 to 440	336 to 422	334 to 420	338 to 430
335 to 441	336 to 423	334 to 421	338 to 431
335 to 442	336 to 424	334 to 422	338 to 432
335 to 443	336 to 425	334 to 423	338 to 433
335 to 444	336 to 426	334 to 424	338 to 434
335 to 445	336 to 427	334 to 425	338 to 435
335 to 446	336 to 428	334 to 426	338 to 436
335 to 447	336 to 429	334 to 427	338 to 437
336 to 406	336 to 430	334 to 428	339 to 403
336 to 407	336 to 431	334 to 429	339 to 404
336 to 408	336 to 432	334 to 430	339 to 405
336 to 409	336 to 433	334 to 431	339 to 406
336 to 410	336 to 434	334 to 432	339 to 407
336 to 411	336 to 435	334 to 433	339 to 408
336 to 412	336 to 436	334 to 434	339 to 409
336 to 413	336 to 437	334 to 435	339 to 410
336 to 414	336 to 438	334 to 436	339 to 411
336 to 415	336 to 439	334 to 437	339 to 412
336 to 416	336 to 440	334 to 438	339 to 413
336 to 417	337 to 404	335 to 402	339 to 414
336 to 418	337 to 405	335 to 403	339 to 415
336 to 419	337 to 406	335 to 404	339 to 416
336 to 420	337 to 407	335 to 405	339 to 417
336 to 421	337 to 408	335 to 406	339 to 418
336 to 422	337 to 409	335 to 407	339 to 419
336 to 423	337 to 410	335 to 408	339 to 420
336 to 424	337 to 411	335 to 409	339 to 421
336 to 425	337 to 412	335 to 410	339 to 422
336 to 426	337 to 413	335 to 411	339 to 423
336 to 427	337 to 414	335 to 412	339 to 424

336 to 428	337 to 415	335 to 413	339 to 425
336 to 429	337 to 416	335 to 414	339 to 426
336 to 430	337 to 417	335 to 415	339 to 427
336 to 431	337 to 418	335 to 416	339 to 428
336 to 432	337 to 419	335 to 417	339 to 429
336 to 433	337 to 420	335 to 418	339 to 430
336 to 434	337 to 421	335 to 419	339 to 431
336 to 435	337 to 422	335 to 420	339 to 432
336 to 436	337 to 423	335 to 421	339 to 433
336 to 437	337 to 424	335 to 422	339 to 434
336 to 438	337 to 425	335 to 423	339 to 435
336 to 439	337 to 426	335 to 424	339 to 436
336 to 440	337 to 427	335 to 425	339 to 437
336 to 441	337 to 428	335 to 426	340 to 403
336 to 442	337 to 429	335 to 427	340 to 404
336 to 443	337 to 430	335 to 428	340 to 405
336 to 444	337 to 431	335 to 429	340 to 406
336 to 445	337 to 432	335 to 430	340 to 407
336 to 446	337 to 433	335 to 431	340 to 408
336 to 447	337 to 434	335 to 432	340 to 409
337 to 406	337 to 435	335 to 433	340 to 410
337 to 407	337 to 436	335 to 434	340 to 411
337 to 408	337 to 437	335 to 435	340 to 412
337 to 409	337 to 438	335 to 436	340 to 413
337 to 410	337 to 439	335 to 437	340 to 414
337 to 411	337 to 440	335 to 438	340 to 415
337 to 412	338 to 404	336 to 402	340 to 416
337 to 413	338 to 405	336 to 403	340 to 417
337 to 414	338 to 406	336 to 404	340 to 418
337 to 415	338 to 407	336 to 405	340 to 419
337 to 416	338 to 408	336 to 406	340 to 420
337 to 417	338 to 409	336 to 407	340 to 421

337 to 418	338 to 410	336 to 408	340 to 422
337 to 419	338 to 411	336 to 409	340 to 423
337 to 420	338 to 412	336 to 410	340 to 424
337 to 421	338 to 413	336 to 411	340 to 425
337 to 422	338 to 414	336 to 412	340 to 426
337 to 423	338 to 415	336 to 413	340 to 427
337 to 424	338 to 416	336 to 414	340 to 428
337 to 425	338 to 417	336 to 415	340 to 429
337 to 426	338 to 418	336 to 416	340 to 430
337 to 427	338 to 419	336 to 417	340 to 431
337 to 428	338 to 420	336 to 418	340 to 432
337 to 429	338 to 421	336 to 419	340 to 433
337 to 430	338 to 422	336 to 420	340 to 434
337 to 431	338 to 423	336 to 421	340 to 435
337 to 432	338 to 424	336 to 422	340 to 436
337 to 433	338 to 425	336 to 423	340 to 437
337 to 434	338 to 426	336 to 424	341 to 403
337 to 435	338 to 427	336 to 425	341 to 404
337 to 436	338 to 428	336 to 426	341 to 405
337 to 437	338 to 429	336 to 427	341 to 406
337 to 438	338 to 430	336 to 428	341 to 407
337 to 439	338 to 431	336 to 429	341 to 408
337 to 440	338 to 432	336 to 430	341 to 409
337 to 441	338 to 433	336 to 431	341 to 410
337 to 442	338 to 434	336 to 432	341 to 411
337 to 443	338 to 435	336 to 433	341 to 412
337 to 444	338 to 436	336 to 434	341 to 413
337 to 445	338 to 437	336 to 435	341 to 414
337 to 446	338 to 438	336 to 436	341 to 415
337 to 447	338 to 439	336 to 437	341 to 416
338 to 406	338 to 440	336 to 438	341 to 417
338 to 407	339 to 404	337 to 402	341 to 418

338 to 408	339 to 405	337 to 403	341 to 419
338 to 409	339 to 406	337 to 404	341 to 420
338 to 410	339 to 407	337 to 405	341 to 421
338 to 411	339 to 408	337 to 406	341 to 422
338 to 412	339 to 409	337 to 407	341 to 423
338 to 413	339 to 410	337 to 408	341 to 424
338 to 414	339 to 411	337 to 409	341 to 425
338 to 415	339 to 412	337 to 410	341 to 426
338 to 416	339 to 413	337 to 411	341 to 427
338 to 417	339 to 414	337 to 412	341 to 428
338 to 418	339 to 415	337 to 413	341 to 429
338 to 419	339 to 416	337 to 414	341 to 430
338 to 420	339 to 417	337 to 415	341 to 431
338 to 421	339 to 418	337 to 416	341 to 432
338 to 422	339 to 419	337 to 417	341 to 433
338 to 423	339 to 420	337 to 418	341 to 434
338 to 424	339 to 421	337 to 419	341 to 435
338 to 425	339 to 422	337 to 420	341 to 436
338 to 426	339 to 423	337 to 421	341 to 437
338 to 427	339 to 424	337 to 422	342 to 403
338 to 428	339 to 425	337 to 423	342 to 404
338 to 429	339 to 426	337 to 424	342 to 405
338 to 430	339 to 427	337 to 425	342 to 406
338 to 431	339 to 428	337 to 426	342 to 407
338 to 432	339 to 429	337 to 427	342 to 408
338 to 433	339 to 430	337 to 428	342 to 409
338 to 434	339 to 431	337 to 429	342 to 410
338 to 435	339 to 432	337 to 430	342 to 411
338 to 436	339 to 433	337 to 431	342 to 412
338 to 437	339 to 434	337 to 432	342 to 413
338 to 438	339 to 435	337 to 433	342 to 414
338 to 439	339 to 436	337 to 434	342 to 415

338 to 440	339 to 437	337 to 435	342 to 416
338 to 441	339 to 438	337 to 436	342 to 417
338 to 442	339 to 439	337 to 437	342 to 418
338 to 443	339 to 440	337 to 438	342 to 419
338 to 444	340 to 404	338 to 402	342 to 420
338 to 445	340 to 405	338 to 403	342 to 421
338 to 446	340 to 406	338 to 404	342 to 422
338 to 447	340 to 407	338 to 405	342 to 423
339 to 406	340 to 408	338 to 406	342 to 424
339 to 407	340 to 409	338 to 407	342 to 425
339 to 408	340 to 410	338 to 408	342 to 426
339 to 409	340 to 411	338 to 409	342 to 427
339 to 410	340 to 412	338 to 410	342 to 428
339 to 411	340 to 413	338 to 411	342 to 429
339 to 412	340 to 414	338 to 412	342 to 430
339 to 413	340 to 415	338 to 413	342 to 431
339 to 414	340 to 416	338 to 414	342 to 432
339 to 415	340 to 417	338 to 415	342 to 433
339 to 416	340 to 418	338 to 416	342 to 434
339 to 417	340 to 419	338 to 417	342 to 435
339 to 418	340 to 420	338 to 418	342 to 436
339 to 419	340 to 421	338 to 419	342 to 437
339 to 420	340 to 422	338 to 420	343 to 403
339 to 421	340 to 423	338 to 421	343 to 404
339 to 422	340 to 424	338 to 422	343 to 405
339 to 423	340 to 425	338 to 423	343 to 406
339 to 424	340 to 426	338 to 424	343 to 407
339 to 425	340 to 427	338 to 425	343 to 408
339 to 426	340 to 428	338 to 426	343 to 409
339 to 427	340 to 429	338 to 427	343 to 410
339 to 428	340 to 430	338 to 428	343 to 411
339 to 429	340 to 431	338 to 429	343 to 412

339 to 430	340 to 432	338 to 430	343 to 413
339 to 431	340 to 433	338 to 431	343 to 414
339 to 432	340 to 434	338 to 432	343 to 415
339 to 433	340 to 435	338 to 433	343 to 416
339 to 434	340 to 436	338 to 434	343 to 417
339 to 435	340 to 437	338 to 435	343 to 418
339 to 436	340 to 438	338 to 436	343 to 419
339 to 437	340 to 439	338 to 437	343 to 420
339 to 438	340 to 440	338 to 438	343 to 421
339 to 439	341 to 404	339 to 402	343 to 422
339 to 440	341 to 405	339 to 403	343 to 423
339 to 441	341 to 406	339 to 404	343 to 424
339 to 442	341 to 407	339 to 405	343 to 425
339 to 443	341 to 408	339 to 406	343 to 426
339 to 444	341 to 409	339 to 407	343 to 427
339 to 445	341 to 410	339 to 408	343 to 428
339 to 446	341 to 411	339 to 409	343 to 429
339 to 447	341 to 412	339 to 410	343 to 430
340 to 406	341 to 413	339 to 411	343 to 431
340 to 407	341 to 414	339 to 412	343 to 432
340 to 408	341 to 415	339 to 413	343 to 433
340 to 409	341 to 416	339 to 414	343 to 434
340 to 410	341 to 417	339 to 415	343 to 435
340 to 411	341 to 418	339 to 416	343 to 436
340 to 412	341 to 419	339 to 417	343 to 437
340 to 413	341 to 420	339 to 418	
340 to 414	341 to 421	339 to 419	
340 to 415	341 to 422	339 to 420	
340 to 416	341 to 423	339 to 421	
340 to 417	341 to 424	339 to 422	
340 to 418	341 to 425	339 to 423	
340 to 419	341 to 426	339 to 424	

340 to 420	341 to 427	339 to 425	
340 to 421	341 to 428	339 to 426	
340 to 422	341 to 429	339 to 427	
340 to 423	341 to 430	339 to 428	
340 to 424	341 to 431	339 to 429	
340 to 425	341 to 432	339 to 430	
340 to 426	341 to 433	339 to 431	
340 to 427	341 to 434	339 to 432	
340 to 428	341 to 435	339 to 433	
340 to 429	341 to 436	339 to 434	
340 to 430	341 to 437	339 to 435	
340 to 431	341 to 438	339 to 436	
340 to 432	341 to 439	339 to 437	
340 to 433	341 to 440	339 to 438	
340 to 434	342 to 404	340 to 402	
340 to 435	342 to 405	340 to 403	
340 to 436	342 to 406	340 to 404	
340 to 437	342 to 407	340 to 405	
340 to 438	342 to 408	340 to 406	
340 to 439	342 to 409	340 to 407	
340 to 440	342 to 410	340 to 408	
340 to 441	342 to 411	340 to 409	
340 to 442	342 to 412	340 to 410	
340 to 443	342 to 413	340 to 411	
340 to 444	342 to 414	340 to 412	
340 to 445	342 to 415	340 to 413	
340 to 446	342 to 416	340 to 414	
340 to 447	342 to 417	340 to 415	
341 to 406	342 to 418	340 to 416	
341 to 407	342 to 419	340 to 417	
341 to 408	342 to 420	340 to 418	
341 to 409	342 to 421	340 to 419	

341 to 410	342 to 422	340 to 420	
341 to 411	342 to 423	340 to 421	
341 to 412	342 to 424	340 to 422	
341 to 413	342 to 425	340 to 423	
341 to 414	342 to 426	340 to 424	
341 to 415	342 to 427	340 to 425	
341 to 416	342 to 428	340 to 426	
341 to 417	342 to 429	340 to 427	
341 to 418	342 to 430	340 to 428	
341 to 419	342 to 431	340 to 429	
341 to 420	342 to 432	340 to 430	
341 to 421	342 to 433	340 to 431	
341 to 422	342 to 434	340 to 432	
341 to 423	342 to 435	340 to 433	
341 to 424	342 to 436	340 to 434	
341 to 425	342 to 437	340 to 435	
341 to 426	342 to 438	340 to 436	
341 to 427	342 to 439	340 to 437	
341 to 428	342 to 440	340 to 438	
341 to 429			
341 to 430			
341 to 431			
341 to 432			
341 to 433			
341 to 434			
341 to 435			
341 to 436			
341 to 437			
341 to 438			
341 to 439			
341 to 440			
341 to 441			

341 to 442			
341 to 443			
341 to 444			
341 to 445			
341 to 446			
341 to 447			
342 to 406			
342 to 407			
342 to 408			
342 to 409			
342 to 410			
342 to 411			
342 to 412			
342 to 413			
342 to 414			
342 to 415			
342 to 416			
342 to 417			
342 to 418			
342 to 419			
342 to 420			
342 to 421			
342 to 422			
342 to 423			
342 to 424			
342 to 425			
342 to 426			
342 to 427			
342 to 428			
342 to 429			
342 to 430			
342 to 431			

342 to 432			
342 to 433			
342 to 434			
342 to 435			
342 to 436			
342 to 437			
342 to 438			
342 to 439			
342 to 440			
342 to 441			
342 to 442			
342 to 443			
342 to 444			
342 to 445			
342 to 446			
342 to 447			
343 to 406			
343 to 407			
343 to 408			
343 to 409			
343 to 410			
343 to 411			
343 to 412			
343 to 413			
343 to 414			
343 to 415			
343 to 416			
343 to 417			
343 to 418			
343 to 419			
343 to 420			
343 to 421			

343 to 422			
343 to 423			
343 to 424			
343 to 425			
343 to 426			
343 to 427			
343 to 428			
343 to 429			
343 to 430			
343 to 431			
343 to 432			
343 to 433			
343 to 434			
343 to 435			
343 to 436			
343 to 437			
343 to 438			
343 to 439			
343 to 440			
343 to 441			
343 to 442			
343 to 443			
343 to 444			
343 to 445			
343 to 446			
343 to 447			
344 to 406			
344 to 407			
344 to 408			
344 to 409			
344 to 410			
344 to 411			

344 to 412			
344 to 413			
344 to 414			
344 to 415			
344 to 416			
344 to 417			
344 to 418			
344 to 419			
344 to 420			
344 to 421			
344 to 422			
344 to 423			
344 to 424			
344 to 425			
344 to 426			
344 to 427			
344 to 428			
344 to 429			
344 to 430			
344 to 431			
344 to 432			
344 to 433			
344 to 434			
344 to 435			
344 to 436			
344 to 437			
344 to 438			
344 to 439			
344 to 440			
344 to 441			
344 to 442			
344 to 443			

344 to 444			
344 to 445			
344 to 446			
344 to 447			

Table 2: Percentage inhibition of sFLT-1 binding to a monoclonal antibody (2B2) after pre-incubation with 50 µg/ml Domain 4 constructs 0, 1, and 2 and 25 µg/ml Domain 4 construct 3 as determined by BIACore analysis.

Sample	% inhibition of sFLT-1
Domain 4 construct 0	5.85 %
Domain 4 construct 1	64.66 %
Domain 4 construct 2	73.35 %
Domain 4 construct 3	44.87 %

CLAIMS

1. A protein consisting of the amino acid sequence of the fourth Ig-like domain of a VEGF receptor, or a variant of said protein that retains the ability to bind to a VEGF receptor.
- 5 2. A protein according to claim 1, wherein said VEGF receptor belongs to either the FLT receptor family or the KDR/FLK receptor family.
3. A protein according to claim 2, wherein said VEGF receptor is the FLT-1 receptor, the KDR/FLK receptor or the FLT-4 receptor.
4. A protein according to any one of the preceding claims, wherein said VEGF receptor is 10 a human VEGF receptor.
5. A protein according to any one of the preceding claims, wherein said protein binds to the fourth Ig-like domain of a VEGF receptor
6. A protein according to any one of the preceding claims that binds to the fourth Ig-like domain of a VEGF receptor with a dissociation constant of 2 μ M or less, preferably, 15 0.2 μ M or less, more preferably 2nM or less, even more preferably, 20pm or less.
7. A protein according to any one of the preceding claims, wherein said fourth Ig-like domain comprises at least residues 344-406 of the full length FLT-1 sequence given in Figure 1, but no more than residues 316-447 of this sequence.
8. A protein according to claim 7 which consists of residues 338-440, 330-429, 330-440 20 or 338-429 of the full length FLT-1 sequence or a variant of this sequence containing one or more amino acid substitutions that do not decrease the binding affinity of the protein for the fourth Ig-like domain of a VEGF receptor.
9. A protein according to any one of claims 1-6, wherein said fourth Ig-like domain comprises at least residues 342-404 of the full length FLK sequence given in Figure 2, 25 but no more than residues 314-440 of this sequence.
10. A protein according to claim 9 which consists of residues 335-435, 328-424, 328-435 or 335-424 of the full length FLK sequence or a variant of this sequence containing one or more amino acid substitutions that do not decrease the binding affinity of the protein for the fourth Ig-like domain of a VEGF receptor.

11. A protein according to any one of claims 1-6, wherein said fourth Ig-like domain comprises at least residues 340 to 402 of the full length KDR sequence given in Figure 3, but no more than residues 312-438 of this sequence.
12. A protein according to claim 11 which consists of residues 333-433, 326-422, 326-433 or 333-422 of the full length KDR sequence or a variant of this sequence containing one or more amino acid substitutions that do not decrease the binding affinity of the protein for the fourth Ig-like domain of a VEGF receptor.
5
13. A protein according to any one of claims 1-6, wherein said fourth Ig-like domain comprises residues 343-403 of the full length FLT-4 sequence given in Figure 4, but no more than residues 315 to 437 of this sequence.
10
14. A protein according to claim 13 which consists of residues 339-437, 329-423, 329-437 or 339-423 of the full length FLT-4 sequence or a variant of this sequence containing one or more amino acid substitutions that do not decrease the binding affinity of the protein for the fourth Ig-like domain of a VEGF receptor.
15. A protein according to any one of the preceding claims that has been genetically or chemically fused to one or more peptides or polypeptides.
15
16. A protein according to claim 15, which comprises repeated amino acid sequences of the fourth Ig-like domain of a VEGF receptor.
17. A protein according to claim 15 or claim 16, fused to a label.
- 20 18. A protein according to any one of the preceding claims, for use as a pharmaceutical.
19. A functional equivalent of a protein according to any one of claims 1-17 that binds to the fourth Ig-like domain of a VEGF receptor.
- 25 20. A functional equivalent according to claim 19, which is an antiidiotypic antibody, a peptide, an oligopeptide, a peptidomimetic compound or a drug molecule, such as a small natural or synthetic organic molecule.
21. Use of a protein according to any one of claims 1-17 or a functional equivalent according to claim 19 in the manufacture of a medicament for the treatment of a disorder whose pathology is dependent upon a VEGF family-mediated pathway.
25
22. A nucleic acid encoding a protein according to any one of claims 1 to 17.

23. A vector comprising a nucleic acid according to claim 22.
24. A host cell comprising a vector according to claim 23.
25. A pharmaceutical composition comprising a protein according to any one of claims 1-17 or a functional equivalent according to claim 19 or 20, in association with a suitable pharmaceutical excipient.
5
26. A method of treating a patient suffering from a disorder whose pathology is dependent upon a VEGF-mediated pathway comprising administering to a patient a therapeutically-effective amount of a protein according to any one of claims 1-17, a functional equivalent according to claim 19 or 20, a nucleic acid according to claim 22, 10 or a pharmaceutical composition according to claim 25.
27. A method according to claim 26, wherein said disorder is an inflammation, psoriasis, rheumatoid arthritis, hemangiomas, leiomyomas, diabetic retinopathy, angiofibromas, endometriosis, macular degeneration, retinal neovascularisation or cancer.
28. A transgenic animal that has been transformed by a nucleic acid molecule according to 15 claim 22 or a vector according to claim 23.
29. A method for inhibiting the dimerisation of a VEGF receptor, comprising bringing the receptor into contact with a protein, or functional equivalent according to any one of claims 1-20.

1/13

FIG. 1

FLT-1

1	MVSYWDTGVLLCALLSCLLLTGSSSGSKLKDPESLKGQTQHIMQAGQTLH	50
51	LQCRGEAAHKWSLPEMVSKESERLSITKSACGRNGKQFCSTLNTAQAN	100
101	HTGFYSCKYLAVPTSKKETESAIYIFISDTGRPFVEMYSEIPEIIHMTE	150
151	GRELVIPCRVTSPNITVTLKKFPLDTLIPDGKRIIWDSRKGFIIISNATYK	200
201	EIGLLTCEATVNCHLYKTNYLTHRQNTIIDVQISTPRPVKLLRGHTLVL	250
251	NCTATTPLNTRVQMTWSYPDEKNKRASVRRRIDQSNSHANIFYSVLTIDK	300
301	MQNKDKGLYTCRVRSGPSFKSVNTSVHIYDKAFITVKHRKQQVLETVAGK	350
351	RSYRLSMKVKAFPSPEVVWLKDGLPATEKSARYLTRGYSIIKDVTEEDA	400
401	GNYTILLSIKQSNVFKNLTATLIVNVKPQIYEKAVSSFPDPALYPLGSRQ	450
451	ILTCTAYGIPQPTIKWFWHPCNHNHSEARCDFCSNNEESFILDADSNMGN	500
501	RIESITQRMAIEGKNKMASTLVVADSRIISGIYICIASNKVGTGRNISF	550
551	YITDVPNGFHVNLEKMPTEGEDLKLSCTVNKFLYRDVTWILLRTVNNRTM	600
601	HYSISKQKMAITKEHSITLNLTIMNVSLQDSGYACRARNVYTGEEILQK	650
651	KKEITIRDQEAPYLLRNLSDHTVAISSLTLDCHANGVPEPQITWFKNNH	700
701	KIQQEPMIILGPGSSTLFIERVTEEDEGVYHCKATNQKGSVESSAYLTQ	750
751	GTSDKSNLELITLTCTCVAATLFWLLTLLIRKMKRSSSEIKTDYLSIIM	800
801	DPDEVPLDEQCERLPYDASKWEFARERLKLGKSLGRGAFGKVVQASAFGI	850
851	KKSPTCRTVAVKMLKEGATASEYKALMTELKILTHIGHHLNVVNLLGACT	900
901	KQGGPLMVIVEYCKYGNLSNYLKSKRDLFFLNKDAALHMEPKKEKMEPGL	950
951	EQGKKPRLDSVTSESFASSGFQEDKSLSDVEEEEDSDGFYKEPITMEDL	1000
1001	ISYSFQVARGMEFLSSRKCIHRDLAARNILLSENNVVKICDFGLARDIYK	1050
1051	NPDYVRKGDTRLPLKWMAPESIFDKIYSTKSDVWSYGVLLWEIFSLGGSP	1100
1101	YPGVQMDEDFCRLREGMRMRAPEYSTPEIYQIMLDCWHRDPKERPRFAE	1150
1151	LVEKLGDLLQANVQQDGKDYIPINAILTGNSGFTYSTPAFSEDFFKESIS	1200
1201	APKFNSGSSDDVRYVNAFKFMSLERIKTFEELLPNATSMFDDYQGDSSTL	1250
1251	LASPMLKRFTWTDSPKASLKIDLRVTSKSKESGLSDVSRPSFCHSSCGH	1300
1301	VSEGKRRFTYDHAELERKIAACSPPPDYNSSVLYSTPPI	1339

2/13

FIG. 2

FLK

1	MESKALLAVALWFCVETRAASVGLTGFHLPPKLSTQKDILTLANTTLQ	50
51	ITCRGQRDLDWLWPNAQRDSEERVLTTECGGGDSIFCKTLTIPRVVGNDT	100
101	GAYKCSYRDVDIASTVVYVYRSPFIASVSDQHGIVYITENKNKTVVI	150
151	PCRGSIISNLNVSLCARYPEKRFVPDGNRISWDSEIGFTLPSYMSIYAGMV	200
201	FCEAKINDETYQSIMYIVVVVGYRIYDVILSPPHEIELSAGEKLVLNCTA	250
251	RTELNVGLDFTWHSPPSKSHKKIVNRDVKPFPGTVAKMFLSTLTIESVT	300
301	KSDQGEYTCVASSGRMIKRNRFTVRVHTKPFIAFGSGMKSLSVEATVGSQV	350
351	RIPVKYLSYPAPDIKWYRNGRPIESNYTMIVGDELTIMEVTERDAGNYTV	400
401	ILTNPISMEKQSHMVSLVVNVPPQIGEKALISPMDSYQYGTMQTLTCTVY	450
451	ANPPLHHIQWYWQLEEACSYRPGQTSPYACKEWRHVEDFQGGNKIEVTKN	500
501	QYALIEGKNKTVSTLVIQAANVSALYKCEAINKAGRGERVISFHVIRGPE	550
551	ITVQPAAPTEQESVSLCTADRNTFENLTWYKLGSQATSVHMGESLTPV	600
601	CKNLDALWKLNGTMFSNSTNDILIVAFQNASLQDQGDYVCSAQDKKTKKR	650
651	HCLVKQLIILERMAPMITGNLENQTTIGETIEVTCPASGNPTPHITWFK	700
701	DNETLVEDSGIVLRDGNRNLTIIRRVRKEDGGLYTCQACNVLGCARAETLF	750
751	IIEGAQEKTNLEVIILVGTAVIAMFFWLLLIVLRTVKRANEDELKTGYL	800
801	SIVMDPDELPLDERCERLPYDASKWEFPRDRKLKGKPLGRGAFGQVIEAD	850
851	AFGIDKTATCKTAVKMLKEGATHSEHRALMSELKILIHIGHHHLNVNLL	900
901	GACTKPGGPLMVIVEFCKFGNLSTYLRGKRNEFPYKSKGARFRQGKDYV	950
951	GELSVDLKRLDSITSSQSSASSGFVEEKSLSDVEEEEASEELYKDFLTL	1000
1001	EHLICYSFQVAKGMEFLASRKCIIHDLAARNILLSEKVVKICDFGLARD	1050
1051	IYKDPDYVRKGDLPLKWMAPETIFDRVYTIQSDVWSFGVLLWEIFSL	1100
1101	GASPYPGVKIDEFCRRLKEGTRMRAPDYTTPEMYQTMLDCWHEDPNQR	1150
1151	PSFSELVEHLGNLLQANAQQDGKDYIVLPMSETLSMEEDSGLSLPTSPV	1200
1201	SCMEEEVCDPKFHNDNTAGISHYLQNSKRKSRPVSVKTFEDIPLLEEPE	1250
1251	VKVI PDDSQTDSCGMVLASEELKTLEDRNKLSPSFGGMPSKSRESVASE	1300
1301	GSNQTSGYQSGYHSDDTTVYSSDEAGLLKMVDAAVHADSGTTLQLTS	1350
1351	CLNGSGPVPAPPPTPGNHERGAA 1373	

3/13

FIG. 3

KDR

1	MESKVLLAVALWLCVETRAASVGLPSVSDLPLRLSIQKDILTIKANTTLQ	50
51	ITCRGQRDLDWLWPNNQSGSEQRVEVTECSDGLFCKTLTIPKVIGNDTGA	100
101	YKCFYRETDLASVIYVYVQDYRSPFIASVSDQHGVVYITENKNKTVVIPC	150
151	LGSISNLNVSLCARYPEKRFVPDGNRISWDSKKGFTIPSYMISYAGMVFC	200
201	EAKINDESYQSIMYIVVVVGYRIYDVVLSPSHGIELSVGEKLVLNCTART	250
251	ELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEMKKFLSTLTIDGVTRS	300
301	DQGLYTCAASSGLMTKNSTFVRVHEKPFVAFGSGMESLVEATVGERVRI	350
351	PAKYLGYPPPEIKWYKNGIPLESNHTIKAGHVLTIMEVSERDTGNYTVIL	400
401	TNPISKEKQSHVSVLVVYVPPQIGEKSЛИSPVDSYQYGTTQTLTCTVYAI	450
451	PPPHHIHWYWQLEEECANEPSQAVSVTPYPCCEWRSVEDFQGGNKIEVN	500
501	KNQFALIEGKNKTVSTLVIQAANVSALYKCEAVNKVGRGERVISFHVTRG	550
551	PEITLQPDMPQTEQESVSLWCTADRSTFENLTWYKLGQPQPLPIHVGELPT	600
601	PVCKNLDTLWKLNATMFSNSTNDILIMELKNASLQDQGDYVCLAQDRKTK	650
651	KRHCVVRQLTVLERVAPTTGNLENQTTSIGESIEVSCASGNPPPQIMW	700
701	FKDNETLVEDSGIVLKDGNRNLTIRRVRKEDEGLYTCQACSVLGCAKVEA	750
751	FFIIEGAQEKTNLEIIIILVGTAVIAMFFWLLVIILRTVKRANGGELKTG	800
801	YLSIVMDPDELPLDEHCERLPYDASKWEFPRDRKLKGKPLRGAFGQVIE	850
851	ADAFGIDKTATCRTAVAKMLKEGATHSEHRALMSELKILIHIGHHLSNVN	900
901	LLGACTKPGGPLMVIVEFCKFGNLSTYLRSKRNEFVPYKTKGARFRQGKD	950
951	YVGAI PVDLKRRLLDSITSSQSSASSGFVEEKSLSDVEEEAPEDLYKDFL	1000
1001	TLEHLICYSFQVAKGMELASRKCIHRDLAARNILLSEKNVVKICDFGLA	1050
1051	RDIYKDPDYVRKGDLPLKWMAPETIFDRVYTIQSDVWSFGVLLWEIFS	1100
1101	LGASPYPGVKIDEFCRRLKEGTRMRAPDYTTPEMYQTMLDCWHGEPSQR	1150
1151	PTFSELVEHGNLLQANAQQDGKDYIVLPISETLSMEEDSGLSLPTSPVS	1200
1201	CMEEEVCDPKFHEDNTAGISQYLQNSKRKSRPVSVKTFEDIPLLEEPEVK	1250
1251	VIPDDNQTDGMVLASEELKTLEDRTKLSPSFGGMVPKSRESVASEGSN	1300
1301	QTSGYQSGYHSDDTTVYSSEEELLKLIEIGVQTGSTAQILQPDSGTT	1350
1351	LSSPPV 1356	

4/13

FIG. 4

FLT4

1	MQRGAALCLRLWLCLGLLDGLVSDYSMTPPTLNITEESHVIDTGDSLIS	50
51	CRGQHPLEAWPGAQEAPATGDKDSEDTGVVRDCEGTDARPYCKVLLHE	100
101	VHANDTGSYVCYYKYIKARIEGTTAASSYVFVRDFEQPFINKPDTLLVNR	150
151	KDAMWVPCLVSIPLGLNVTLRSQSSVLWPDGQEVVWDRRGMLVSTPLLHD	200
201	ALYLQCETTWGDQDFLSNPFLVHTGNELYDIQLLPRKSLELLVGEKLV	250
251	NCTVWAEFNSGVTFDWDYPGKQAERGKWPERRSQQTTELSSILTIHNV	300
301	SQHDLGSYVCKANNGIQRFRESTEVIVHENPFISVEWLKGPILEATAGDE	350
351	LVKLPVKLAAYPPPEFQWYKDGKALSGRHSPhALVLKEVTEASTGTYTLA	400
401	LWNSAAGLRRNISLELVNVNPPOIHEKEASSPSIYSRHSRQALTCTAYGV	450
451	PLPLSIQWHWRPWTCPCKMFAQRSLRRQQDLMPCRDWRRAVTTQDAVNP	500
501	IESLDTWTEFVEGKNKTVSKLVIQANANVSAMYKCVVSNKVGQDERLIYFY	550
551	VTTIPDGFTIESKPSEELLEGQPVLLSCQADSYKYEHLRWYRLNLSTLHD	600
601	AHGNPLLLDCKNVHLFATPLAASLEEVAPGARHATLSLSIPRVAPEHEGH	650
651	YVCEVQDRRSHDKHCHKKYLSVQALEAPRLTQNLTDLNVNVSDSLEMQCL	700
701	VAGAHAPSIVWYKDERLLEEKSGVDLADSNQKLSIQRVREEDAGPYLCV	750
751	CRPKGCVNSSASAVAVEGSEDKGSMEIVILVGTGVIAVFFWLLLLIFCNM	800
801	RRPAHADIKTGYLSIIMDPGEVPLLEEQCEYLSYDASQWEFPRERLHLGRV	850
851	LGYGAFGVVEASAFGIHKGSSCDTVAVKMLKEGATASEQRALMSELKIL	900
901	IHIGNHNVNVNLLGACTKPQGPLMVIVEFCKYGNLSNFLRAKRDAFSPCA	950
951	EKSPEQRGRFRAMVELARLDRRPGSSDRVLFARFSKTEGGARRASPDQE	1000
1001	AEDLWLSPLTMEDLVCYSFQVARGMEFLASRKCIHRDLAARNILLSESDV	1050
1051	VKICDFGLARDIYKDPDYVRKGSARLPLKWMAPESIFDKVYTTQSDVWSF	1100
1101	GVLLWEIFSLGASPYPGVQINEEFCQRVRDGTRMRAPELATPAIRHIMLN	1150
1151	CWSGDPKARPAFSDLVEILGDLLQGRGLQEEEVCMAPRSSQSSEEGSFS	1200
1201	QVSTMALHIAQADAEDSPPSLQRHSLAARYYNWVSFPGCLARGAETRGSS	1250
1251	RMKTFEFPMTPTTYKGVDNQTDGMVLASEEFEQIESRHRQESGFR	1298

5/13

FIG. 5

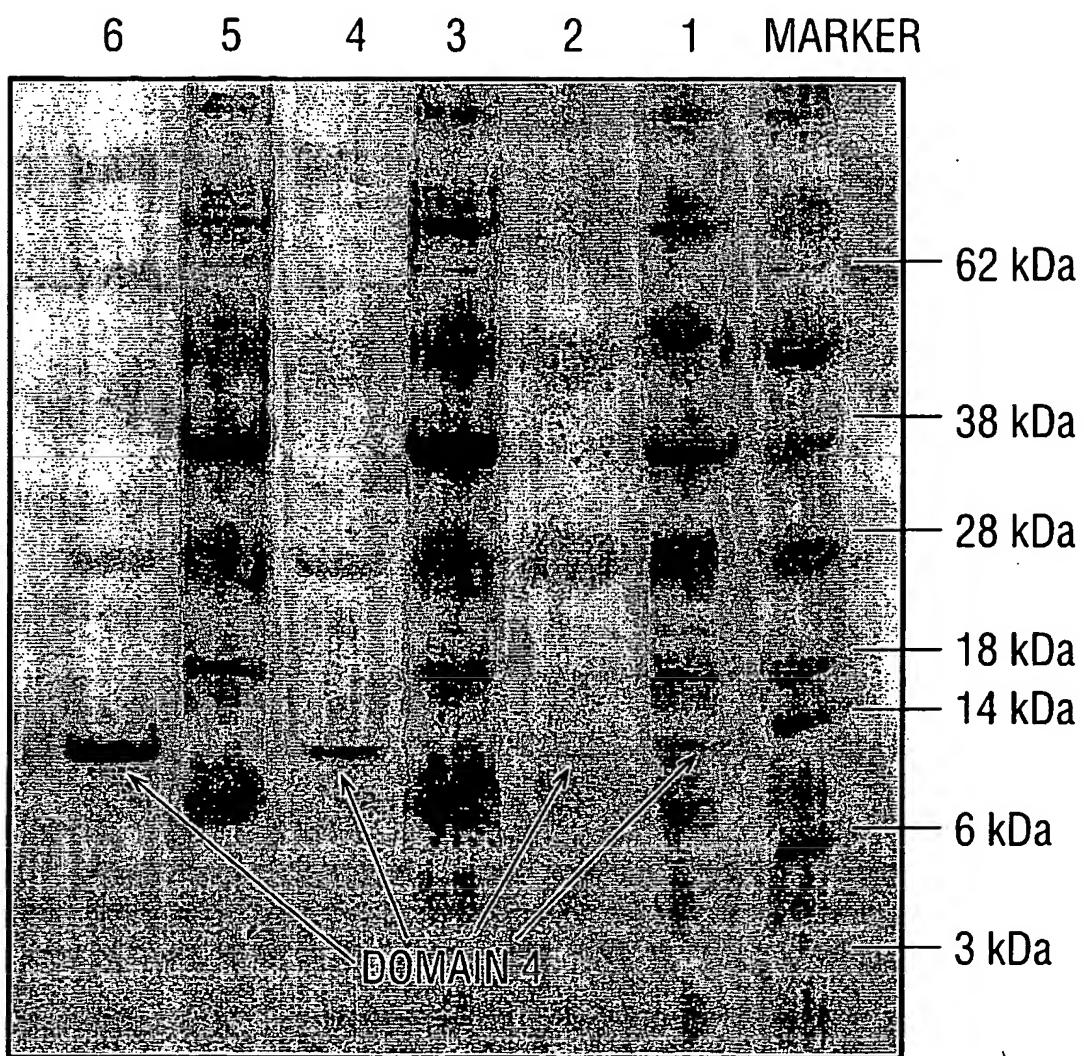
FLT	316	GPSFKSVNTSVHIIY 330DKAFITVKHRKQQVLE- <u>TVAGKRSYRLSMKVKA</u> FPSPEVWLKDGLPATEKSARYLTR	GYSLIIKDVTEEDAGNYTILL--SI---KQSNVFKNLTTATLIVNVKPQIYEKAVSSFPD 440 PALLYPLG447
KDR	312	GLMTKJNISTFVRVH 326 <u>EKPFAFGSGMESILVEATV</u> -GER-VRIPAKYLGYPPPEIKWYKNGIP-LESN-HTIKA	GHVLTIMEVSERDTGNYTVILTNPISKEKQSHVW-----SLVVVYVPPQIGEKSLLISPVDF 433 SYQY--G438
FLK	314	GRMIKRNRRTFVRVH 328TKPFI A FGSGMKSILVEATV-GSQ-VRIPVKYLSYPAPDIKWYRNGRP-IESNYTMI-V	GDELTIMEVTERDAGNYTVILTNPISMEKQSHMV-----SLVVNVPPQIGEKLISPMDF 435 SYQY--G440
FLT4	315	GIQRFRESTEVIVH 329ENPFISVEWLKGPI <u>LEATA</u> -GDELVKL P VKLAAYPPPEFQWYKDG-----KALSGRHS	PHALVLKEVTEASTGTYTLA WNSAAGLR RNTISLELVNVPPQI <u>THEKEASSPS</u> - 433 IYSR---437

Underlined:

Construct 0

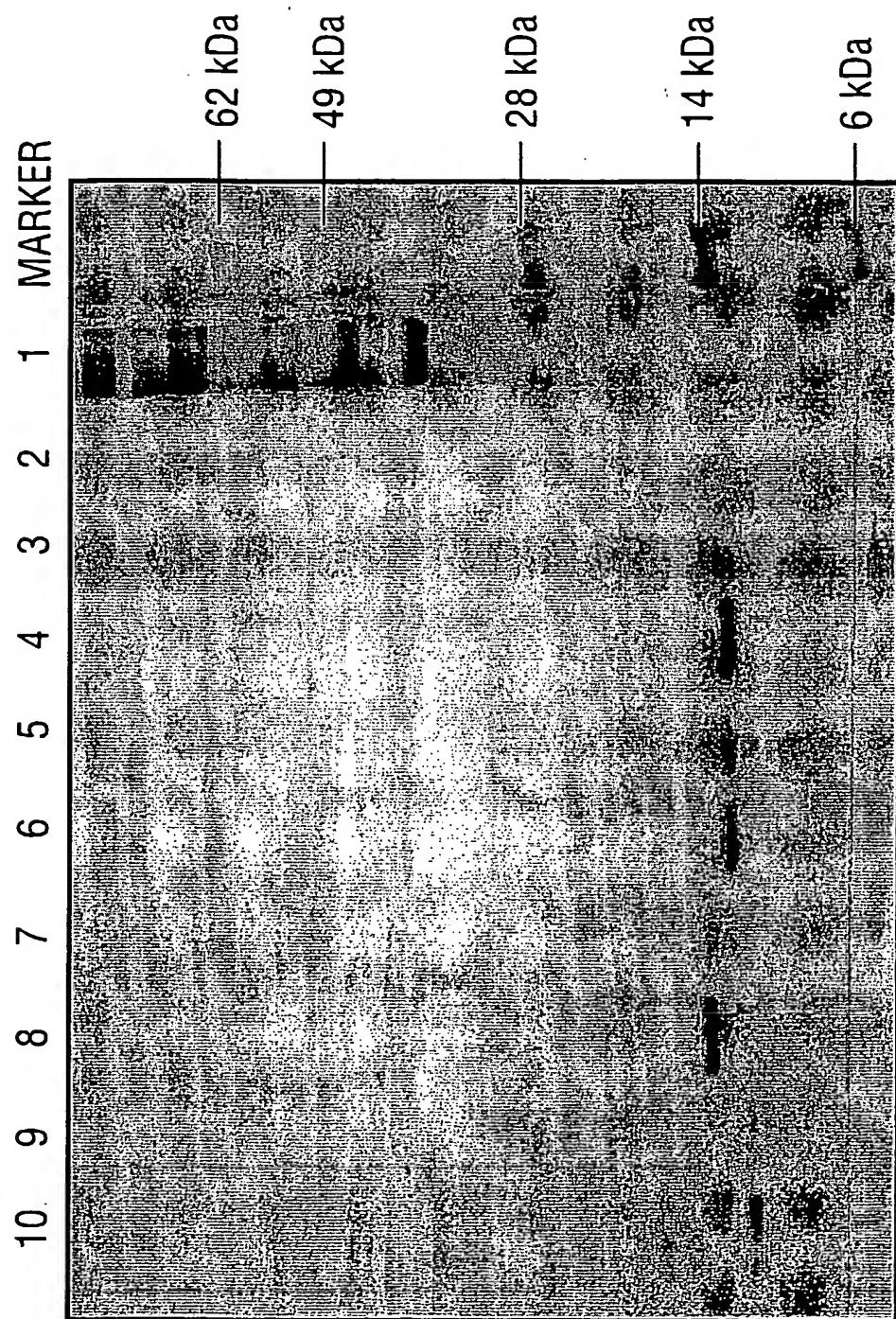
6/13

FIG. 6



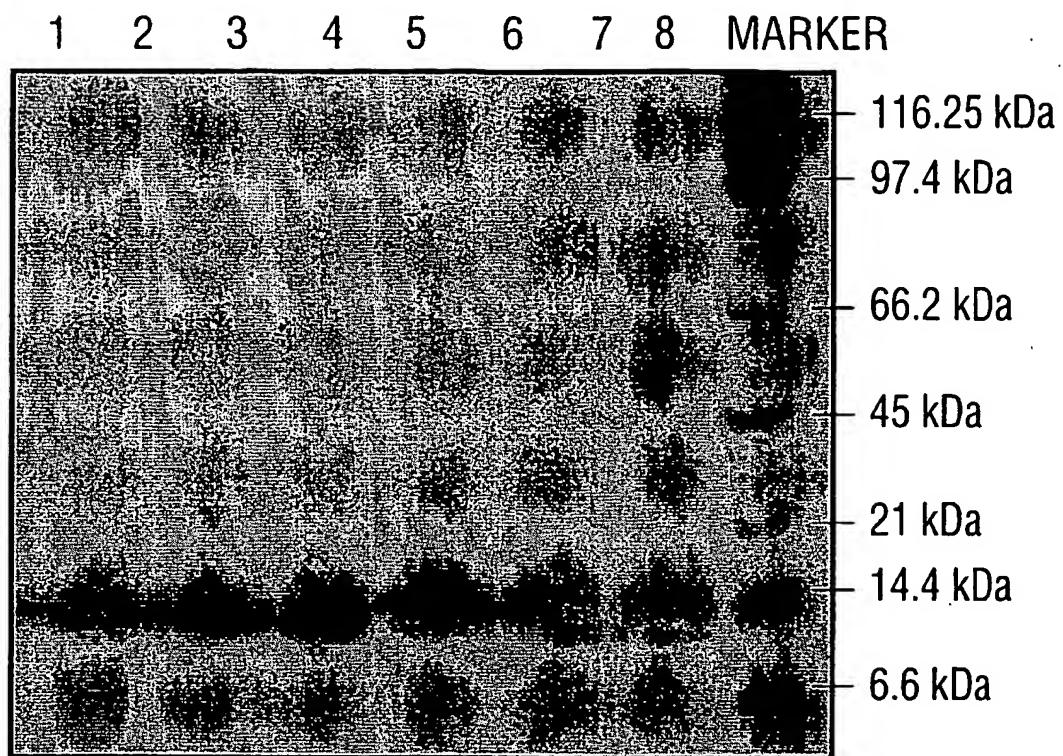
7/13

FIG. 7

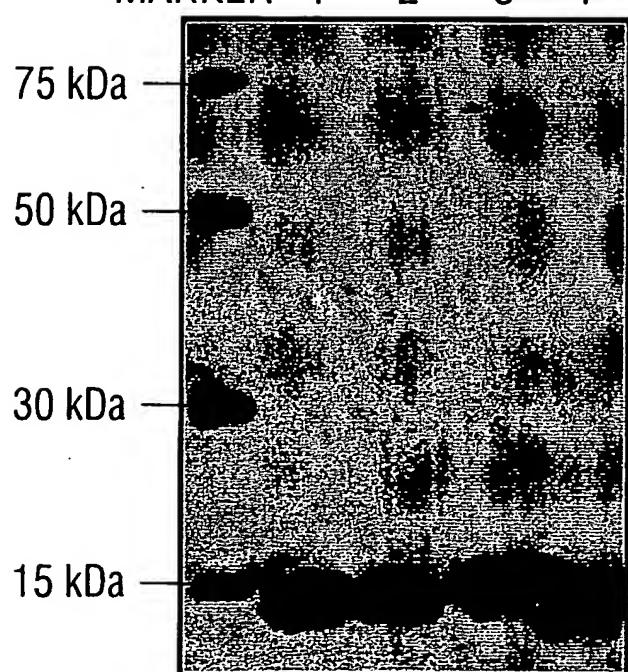


8/13

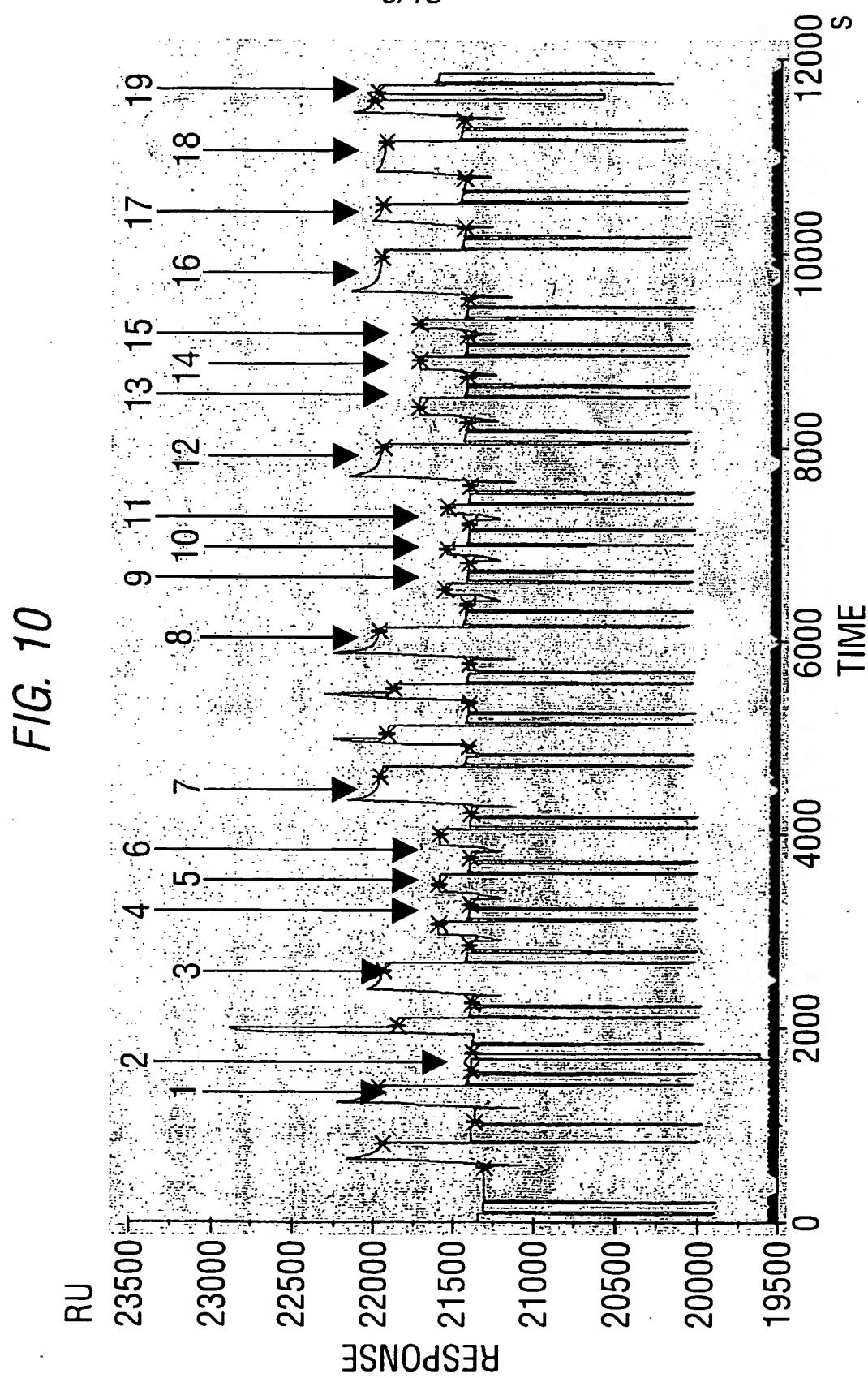
FIG. 8



MARKER 1 2 3 4



9/13



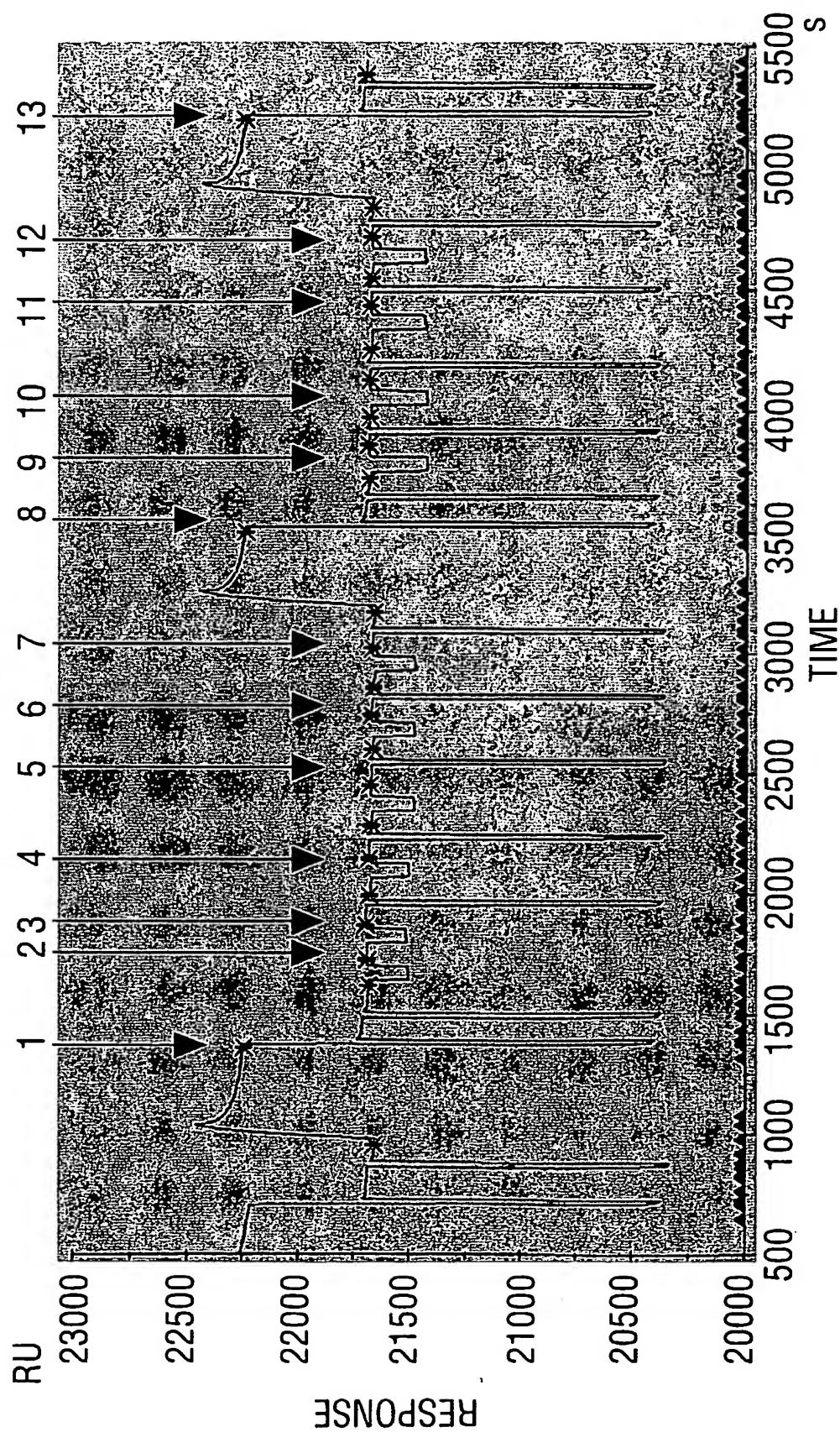
10/13

FIG. 10(*contd.*)

Number from Figure 5	50 nM sFLT	20 mM Tris- HCl, pH7.4	50 nM sFLT + 50 µg/ml D4 (0)	50 nM sFLT + 50 µg/ml D4 (1)	50 nM sFLT + 50 µg/ml D4 (2)	50 nM sFLT + 25 µg/ml D4 (3)
1	540					
2		- 10.9				
3			553.8			
4				194.3		
5					195.6	
6						193.2
7	566.8					137.2
8	561.5					138.9
9						138.1
10						
11						
12	545.3					
13						306
14						300.5
15						303.2
16	535.2					
17					514.3	
18					489.8	
19	555					

11/13

FIG. 11



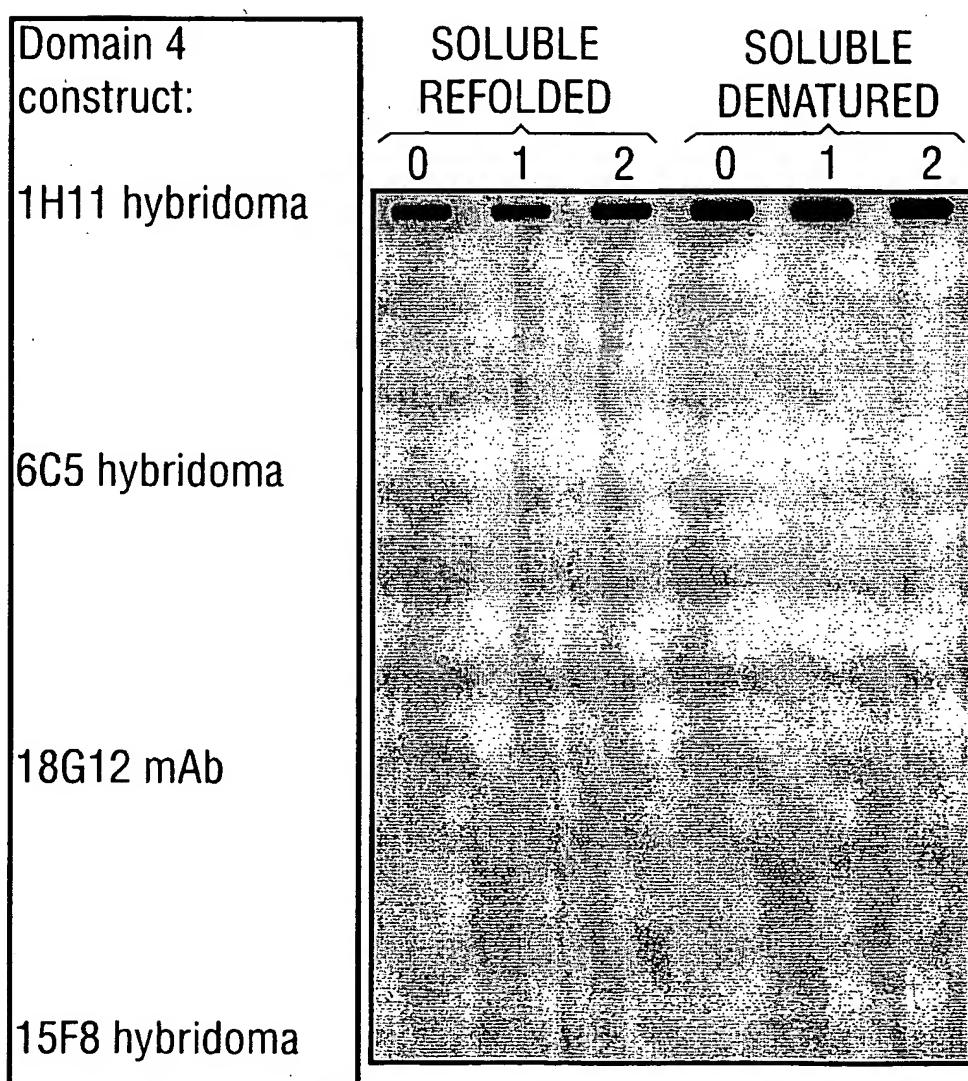
12/13

FIG. 11 (contd.)

Number from Figure 1	50 nM sFLT	50 µg/ml D4 (0)	50 µg/ml D4 (1)	50 µg/ml D4 (2)	25 µg/ml D4 (3)
1	584.8				
2		4.7			
3		13.1			
4		0.7			
5			3.1		
6			5		
7			5.9		
8	578.1				
9				3.3	
10				1.8	
11					2.1
12					-1
13	569.3				

13/13

FIG. 12



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
14 June 2001 (14.06.2001)

PCT

(10) International Publication Number
WO 01/42284 A3

(51) International Patent Classification⁷: C12N 15/12. (72) Inventor; and
15/10, 15/62, 15/66, C07K 14/71, 16/42, A61K 38/18. (75) Inventor/Applicant (for US only): PAPPA, Helen
31/70 Road, Winnersh, Wokingham, Berkshire RG41 5TU (GB).

(21) International Application Number: PCT/GB00/04693 (74) Agents: MERCER, Christopher, Paul et al.; Carpmaels
(22) International Filing Date: 7 December 2000 (07.12.2000) & Ransford, 43 Bloomsbury Square, London WC1A 2RA
(25) Filing Language: English (GB).

(26) Publication Language: English (81) Designated States (national): AE, AG, AL, AM, AT, AU,
(30) Priority Data: 9928950.6 7 December 1999 (07.12.1999) GB AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,
PCRGISNINVLNVSCLCARYPEKRFVPGNRIWSDSEIGFTLPSYMSYAGMV DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,
FCEAKINDETYQSMIYIVVVGYRIVDVLSPSPHEIELSAGEKLVLNCTA HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,
RTELNVGLDFTWHSPPSKSHKKIVNRDVKPFPGTVAKMFLSTLTIESVT LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,
KSDQGEYTCVASSGRMIRKNRTFVRVHTKPPFIAFGSGMKSILVEATVGSQV NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,
(71) Applicant (for all designated States except US): METRIS (84) Designated States (regional): ARIPO patent (GH, GM,
THERAPEUTICS LIMITED [GB/GB]; 515 Eskdale KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
Road, Winnersh, Wokingham, Berkshire RG41 5TU (GB).

[Continued on next page]

(54) Title: PROTEIN COMPRISING IG-LIKE DOMAIN 4 OF A VEGF RECEPTOR MEDIATES RECEPTOR DIMERIZATION.
BUT NOT ACTIVATION

PLK	
1	MESKALLAVALWFVCVETRAASVGLTGDPLHPPKLSTQKDILTLANTTLQ
51	ITCRGQRDI.DWLWPNQAQRDSEERVLVTECGGGDSIFCKTLTIPRVRVGNNDT
101	GAYKCSYRDVDIASIVVVYVRDYSRPFIASVSDQHGIIVYVITENQNKTVV
151	PCRGISNINVLNVSCLCARYPEKRFVPGNRIWSDSEIGFTLPSYMSYAGMV
201	FCEAKINDETYQSMIYIVVVGYRIVDVLSPSPHEIELSAGEKLVLNCTA
251	RTELNVGLDFTWHSPPSKSHKKIVNRDVKPFPGTVAKMFLSTLTIESVT
301	KSDQGEYTCVASSGRMIRKNRTFVRVHTKPPFIAFGSGMKSILVEATVGSQV
351	RIPVKYLSPAPDIXWYRNGRPIESNYTMIVGDELTIMETERDAGNYTV
401	ILTNPISMEKQSHMMSLVLNNVPPQIGEKALISPMDSYQYGTMQTLTCTVY
451	ANPPLHHIQWYVQLEEACSYRPGQTSPYACKEWHRHVEDPQGGNKKIEVTKN
501	QYALIEGKNTVSTLVIQAAANVSALYKCEAINKAGRGERVISFHVIRGPE
551	ITVQPAAPTEQESVSLLCTADRNFENLTWYKLGQSATSVHMGESLTPV
601	CKNLDALWKLNGTMFSNSTKDLIIVAFQNAQLQDOGDDYVCSAQDKKTKKR
651	HCLVKQLIILERMAPMTGNLENQTTTGETIEVTCPASGNPTPB1TMFK
701	DNETLVEDSGIVLRDGNRNLTIIRRVRKEDGGLYTCCACNVLCCARAETLF
751	IIEGAQEKTNLEVILVLTGAVIAMFFWLLVIVLRTVKRANEGETKTYL
801	SIVMDPDELPLDRCERCLPYDASKWEPFRDRLKLGPPLGRGAFQVIEAD
851	AFGIDKTKTAKTVAVKMLKEGATHSEHRAIMSELKILIHGHHLNVVNLL
901	GACTKPGGPLMIVVEPKFGNLSTYLRGKRNEFVPPYKSKGARFRQGKDYY
951	GELSVDLKRLDSITSSQSSASSGFVEEKSLSDVEEEASEEELYKDFLTL
1001	EHLICYSFQVAKGMEFLASRKCIHRDIAARNVLLSEKNVVKICDFGLARD
1051	IYKDPDVYRKGEARLPLKWMAPETIFDRVYTTIQS3WNSPGVLLWEIFSL
1101	GASPYPGVKIDEEFCRRLKEGTRMRAPDYTTPEMYQTMDCWHEDPHQR
1151	PSFSELVEHILQULLQANAQCDGKDYIVLPMSETLSMEEDSGLSLPTSPV
1201	SCHEEEEVCDPKFYDNTAGISHYLCNSKRKSPPVSVKTFEDIPLLEPPE
1251	VKVIPODSQTDEGMVLASEELKTLEDRNKLSPSPGGMMPSKSRESVASE
1301	GSKOTSGYQSGYHSDDTDTTVYSSD2AGLIMVDAVHADSGTTIQLTS
1351	CLNGSGPVPAPPPTPGNHERGAA 1373

(57) Abstract: The invention relates to novel compounds that act to prevent dimerisation of vascular endothelial growth factor (VEGF) receptors. The novel compounds may comprise the amino acid sequence of the fourth Ig-like domain of a VEGF receptor, or a variant that retains the ability to bind to a VEGF receptor. These compounds are useful in the inhibition of the biological activity of VEGF receptors and may thus be used to treat diseases in which VEGF plays a role.

WO 01/42284 A3



patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR). OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

(88) Date of publication of the international search report:
10 May 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/04693

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7	C12N15/12	C12N15/10	C12N15/62	C12N15/66	C07K14/71
	C07K16/42	A61K38/18	A61K31/70		

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>TANAKA KEIJI ET AL: "Characterization of the extracellular domain in vascular endothelial growth factor receptor-1 (Flt-1 tyrosine kinase)." JAPANESE JOURNAL OF CANCER RESEARCH, vol. 88, no. 9, 1997, pages 867-876, XP001015447 ISSN: 0910-5050 page 875, left-hand column, paragraph 3</p> <p>---</p> <p>-/-</p>	15, 17-19, 22-24

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority (claims) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

8 August 2001

22/08/2001

Name and mailing address of the ISA
European Patent Office, P.O. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel (+31-70) 340-2040, Tx. 31 651 epo nl
Fax: (+31-70) 340-3016

Authorized officer

Smalt, R

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/04693

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>SHINKAI AKEO ET AL: "Mapping of the sites involved in ligand association and dissociation at the extracellular domain of the kinase insert domain-containing receptor for vascular endothelial growth factor" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 273, no. 47, 20 November 1998 (1998-11-20), pages 31283-31288, XP002166839 ISSN: 0021-9258 figure 1 ---</p>	15, 17-19, 22-24
X	<p>KENDALL R L ET AL: "IDENTIFICATION OF A NATURAL SOLUBLE FORM OF THE VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR, FLT-1, AND ITS HETERO-DIMERIZATION WITH KDR" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, US, ACADEMIC PRESS INC. ORLANDO, FL, vol. 226, no. 2, 13 September 1996 (1996-09-13), pages 324-328, XP000611908 ISSN: 0006-291X cited in the application the whole document ---</p>	15, 17-19, 22-24
X	<p>WO 94 21679 A (KENDALL RICHARD L ; THOMAS KENNETH A JR (US); MERCK & CO INC (US)) 29 September 1994 (1994-09-29) figure 1 ---</p>	15, 17-19, 22-24
Y	<p>BARLEON BERNHARD ET AL: "Mapping of the sites for ligand binding and receptor dimerization at the extracellular domain of the vascular endothelial growth factor receptor FLT-1." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 16, 1997, pages 10382-10388, XP002174230 ISSN: 0021-9258 cited in the application the whole document --- -/-</p>	1-29

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/04693

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KENDALL ET AL: "Inhibition of vascular endothelial cell growth factor by an endogenously encoded soluble receptor" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, US, vol. 90, no. 22, 15 November 1993 (1993-11-15), pages 10705-10709, XP002146190 ISSN: 0027-8424 cited in the application the whole document ---	1-29
A	WO 97 44453 A (CHEN HELEN HSIFEI ;FERRARA NAPOLEONE (US); GENENTECH INC (US); PRE) 27 November 1997 (1997-11-27) the whole document ---	
A	WO 95 33050 A (SHARKEY ANDREW MARK ;SMITH STEPHEN KEVIN (GB); LYNXVALE LTD (GB);) 7 December 1995 (1995-12-07) the whole document ---	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 00/04693

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9421679	A 29-09-1994	AU 684498	B	18-12-1997
		AU 6393494	A	11-10-1994
		CA 2158745	A	29-09-1994
		EP 0694042	A	31-01-1996
		JP 8508161	T	03-09-1996
		US 5712380	A	27-01-1998
		US 5861484	A	19-01-1999
WO 9744453	A 27-11-1997	US 6100071	A	08-08-2000
		AU 717112	B	16-03-2000
		AU 3060497	A	09-12-1997
		EP 0907733	A	14-04-1999
		JP 2000502357	T	29-02-2000
		NZ 332779	A	29-06-1999
		US 5952199	A	14-09-1999
WO 9533050	A 07-12-1995	AU 695393	B	13-08-1998
		AU 2535495	A	21-12-1995
		CA 2191071	A	07-12-1995
		CN 1159827	A	17-09-1997
		EP 0783571	A	16-07-1997
		JP 10504183	T	28-04-1998
		NZ 285834	A	28-10-1998
		US 6011003	A	04-01-2000

BEST AVAILABLE COPY